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Epidemiology and Control of cattle ticks and tick-borne infections in Central Nigeria

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Declaration

I declare that the research described within this thesis is my own work and that this thesis is my own composition and I certify that it has never been submitted for any other degree or professional qualification.

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Abstract

Cattle ticks and tick-borne infections (TBIs) undermine cattle health and productivity in the whole of sub-Saharan Africa (SSA) including Nigeria. In this West African country, two thirds of the cattle population are reared in the central-northern regions, kept under the traditional pastoral husbandry of Fulani herders. Under the Fulanis' management, cattle are grazed extensively, being exposed to infestation by several tick genera (i.e. *Amblyomma*, *Hyalomma*, and *Rhipicephalus* spp., sub-genus *Boophilus* spp. included), vectors of the causative agents of the most important bovine TBIs in West Africa: anaplasmosis, babesiosis and ehrlichiosis (cowdriosis). Nevertheless, the Fulani pastoralists do not usually employ chemicals to control ticks in their cattle, merely relying on traditional methods (i.e. manual removal of the most conspicuous specimens). This approach, however, does not prevent cattle from being re-infested, leaving the animals challenged by a broad variety of other tick species, most of which are vectors of economically relevant TBIs.

Knowledge of tick and TBIs occurrence is an essential pre-requisite to assist field diagnosis and devising effective control strategies for a given area. Existing information on tick infestation of cattle in Nigeria is rather out-dated, mostly derived from studies carried out in the south of the country. Similarly, all studies published to date on cattle TBIs in the country do not include any molecular analysis, being based instead on cytological and/or serological diagnostics. Therefore, the aim of the present thesis was to assess the presence of cattle ticks and TBIs occurring in an area of Central Nigeria (i.e. Plateau State). This is a densely populated area with traditionally managed cattle, where no acarides have historically been employed on livestock.

The work undertaken herein firstly reviews the information available to date on ticks and TBIs known to be endemic in Nigerian cattle, identifying gaps present in the existing knowledge, leading to the rationale of this study. An initial survey was conducted documenting the tick species infesting cattle in Central Nigeria, in

order to assess the infestation rate of surveyed animals at the time of the year when the tick load on the host is known to be most abundant (i.e. the wet season). The survey provided novel information on tick populations in cattle in Nigeria disclosing the presence of a broad variety of species, most of which are vectors of hazardous TBIs.

In order to conduct a molecular diagnosis of the TBIs within the study area, a novel methodology was developed (i.e. reverse line blotting, RLB). The application of this approach was based on a thorough review of its application to the diagnosis of TBIs worldwide as well as in SSA. The optimisation of the RLB at the University of Edinburgh to enable the detection of a broad-spectrum of TBIs in Nigeria, caused by an array of five genera of microorganisms (i.e. *Ehrlichia* and *Anaplasma*, *Theileria* and *Babesia*, *Rickettsia* spp.) is presented. The assessment of the analytical sensitivity of this technique for the detection of *Anaplasma marginale*, a highly endemic tick-borne pathogen in SSA, demonstrated a detection threshold of ≥ 7 infected cells (keeping the limit of a natural infection).

The occurrence of TBIs in cattle in the study area was assessed during a large-scale epidemiological survey through the application of the validated RLB. This study disclosed the occurrence of a high prevalence of several bovine TBIs in Central Nigeria, some of which are of great veterinary and zoonotic concern. The RLB enabled the detection of carrier status as well as of numerous multiple infections (69.5%, 95% CI: 65.5–73.6%). Based on the findings presented, endemic stability for highly prevalent haemoparasites (i.e. *Theileria mutans*, *Theileria velifera*, *Theileria taurotragi*, *Anaplasma marginale*, *Ehrlichia* species Omatjenne) is postulated, whereas a more instable epidemiological scenario is hypothesized for other microorganisms (i.e. *Anaplasma centrale* and *Babesia bovis*), which might be connected with outbreaks of clinically apparent disease, sporadically seen in the study area.

The effect of a monthly tsetse-borne trypanosomiasis-focused control programme (based on the application 0.005% deltamethrin spray formulation,

applied only to the lower quarters of cattle) on the kinetics of bovine TBIs was assessed at the village level. Longitudinal monitoring of control and treated cattle was conducted over the period of eleven months. Results generated provide input to the improvement of future control strategies to be rolled out across SSA, aiming to achieve an integrated control of both trypanosomiasis and TBIs.

The present thesis contributes to a better understanding of the epidemiology of bovine TBIs in Nigeria as well as in the rest of West Africa, using a highly sensitive tool of wide applicability. These findings will be shared with the local pastoralist communities to further promote effective yet sustainable, vector control, in tune with the traditional long-established practices.

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List of abbreviations

AAAP – Attraction-aggregation attachment pheromone

AAT – African animal trypanosomiasis

AO – Arsenious oxide

ATBF – African tick-bite fever

BBSRC – Biotechnology and Biological Sciences Research Council

BHC – Benzenehexachloride

BLAST – Basic Local Alignment Search Tool

CI – Cumulative incidence

CIDLID – Combating Infectious Diseases in Livestock for International Development

DDT – Dichlorodiphenyltrichloroethane

DNA – Deoxyribonucleic acid

DPI – Days post-infection

ECf – East Coast fever

EDAC – 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-liked immunosorbent assay

EMBO – European Microbiology Organization

EVPC – European Veterinary Parasitology College

FAO – Food and Agriculture Organization (of the United Nations)

FRET – Fluorescence resonance energy transfer

ICTDD – International Consortium on Ticks and Tick-borne Diseases

ICONZ – Integrated Control Of Neglected Zoonoses

IFAT – Indirect fluorescent antibody test

IPM – Integrated Pest Management

ISF – Israeli Spotted Fever

ITM – Infection-and-Treatment Method

LGA – Local Government Area

LBW – Live body weight

mLAMP – Multiplex loop-mediated isothermal amplification

mPCR – Multiplex polymerase chain reaction

NITR – Nigerian Institute for Trypanosomiasis Research

NVRI – Nigerian Veterinary Research Institute

OPs – Organophosphates

PCR – Polymerase chain reaction

qPCR – Quantitative polymerase chain reaction

RH – Relative humidity

RDT – Rapid diagnostic test

RLB – Reverse line blotting

rRNA – Ribosomal ribonucleic acid

SE – Standard error

SFG – Spotted fever group

SSA – Sub-Saharan Africa

SOS – Stamp Out Sammore

TBIs – Tick-borne infections

TBDs – Tick-borne diseases

TIBOLA – Tick-borne lymphoadenopathy

UCTD – Utrecht Centre for Tick-borne Diseases

VBDs – Vector-borne diseases

VPRG – Veterinary Parasite Resistance Group

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Chapter 1 – Introduction and Literature Review

1.1 Introducing the research focus

Ticks constitute a major limiting factor to livestock production in many parts of the world, including sub-Saharan Africa (SSA) (Jongejan and Uilenberg, 2004). In much of Africa, as well as in other sub-tropical and tropical areas of the world, numerous tick species have long been established (Amoo, 1992; Punya, 1992) (Figure 1.1).

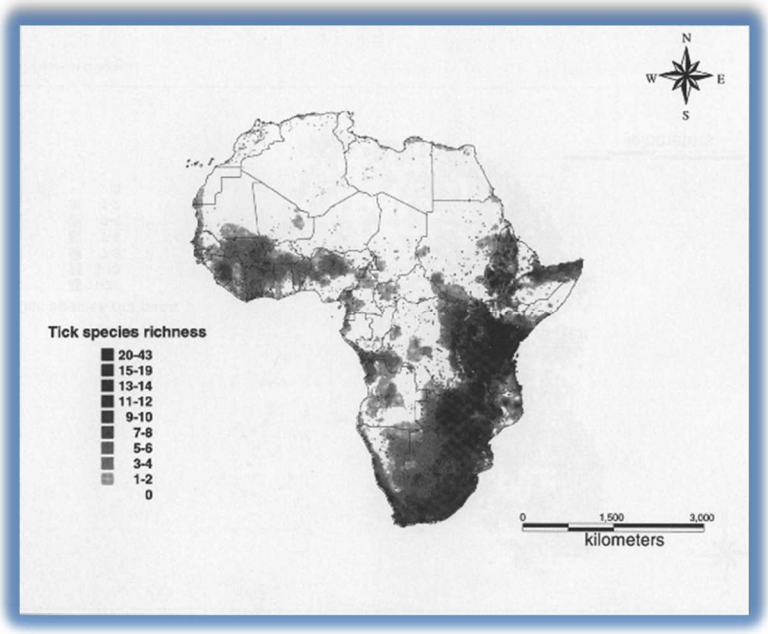

Figure 1.1 – Augmented estimate of pan-African tick species richness (from Cumming, 2000),

prepared using tick collection data obtained from 34,060 individual locality records assembled from sources published over the period 1900–1997. Species range estimates at 0.25 x 0.25-degree resolution were produced using logistic regression based on climatic factors (i.e. mean monthly maximum temperature; minimum temperature, rainfall and elevation) and normalized difference vegetation index. More details on the sources consulted are provided in Cumming (1999).

Here, the importation of exotic European and North-American taurine (i.e. *Bos taurus*) cattle breeds in the late nineteenth Century caused the aggravation of the health problems resulting from both the direct and indirect harmfulness of tick infestation in cattle, due to the higher susceptibility of these breeds compared to autochthonous (i.e. *Bos indicus*) cattle (Uilenberg, 1995; George *et al.*, 2004).

In Nigeria, two thirds of the cattle population are concentrated in the central-northern part of the country, mostly reared according to the traditional pastoral husbandry of the Fulani herders (Awogbade, 1979).

Under the Fulani management, cattle are extensively grazed in pastures and forested areas, being exposed to infestation by ticks of the three genera present in Nigeria (i.e. *Amblyomma*, *Hyalomma*, and *Rhipicephalus* spp., sub-genus *Boophilus* spp. included) (Awogbade, 1979; Dipeolu, 1975a; Pullan, 1980; Bayer and Maina, 1984); including species known to act as vectors of the causative agents of the most important bovine tick-borne infections (TBIs) in West Africa, namely anaplasmosis, babesiosis, cowdriosis (ehrlichiosis) and theileriosis (Leeflang and Ilemobade, 1977a,b).

These TBIs are known for being endemic in the indigenous (*B. indicus*) cattle population (Leeflang and Ilemobade, 1977a,b; Iwuala and Okpala, 1978; Obi, 1978), in which they are responsible for chronic rather than acute conditions. Losses due to TBIs in these animals are mainly due to the gradual deterioration of their health status rather than acute outbreak of disease (Maina, 1986). Nevertheless, clinically apparent tick-borne diseases (TBDs) may still occur in indigenous cattle in particular circumstances of malnutrition or debilitation by a concurrent pathological condition (e.g. trypanosomiasis) (Awogbade, 1979; Maina, 1986), or during the wet season, in the presence of high tick challenge (Bayer and Maina, 1984).

Tick burdens on cattle tend to increase especially after the first scattered rains, reaching the highest abundance one month after the heavy rains (i.e. from July to September), when all tick species are expected to be present (Bayer and Maina, 1984; Maina, 1986; Pukuma *et al.*, 2011). These are the times of the most frequent instances when the Fulani pastoralists seek veterinary assistance to provide their animals with the necessary therapeutic treatment (Maina, 1986). Alternatively, when veterinary professionals are out of reach, the Fulani herders occasionally intervene autonomously, administering the drugs they purchased in agro-vet shops (Tok[1],

[1] Dr Tok, Langs; field veterinarian at the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos, Plateau State, Nigeria.

personal communication). This may result in the improper management of clinical diseases, with the risk of under- or over-dosing anti-microbial (e.g. oxytetracycline in different concentrations) and trypanocidal drugs (e.g. dimenazene or a combination of the former with isometamidium) (Majekodunmi, 2011). Besides hampering the correct diagnosis of diseases, the improper use of these drugs might also lead in the long run to the appearance of chemoresistance (Eisler *et al.*, 1997; Geerts *et al.*, 2001).

Furthermore, TBIs also represent a major limitation to the improvement of cattle production given the high morbidity and mortality rates they can cause in more productive, but susceptible, exotic (*B. taurus*) cattle breeds, when brought into the area for crossbreeding purposes (Ajayi *et al*., 1982).

Knowledge of tick distribution as well as the occurrence of the infections that these arthropods may transmit is an essential pre-requisite for devising any effective control of these ectoparasites on cattle as well the environment in a target area (de Castro, 1997; Walker, 2011). With respect to Nigeria, existing information on cattle ticks and TBIs is outdated and mostly refers to the southern part of the country (Dipeolu, 1975b; Leeflang and Ilemobade, 1977a,b; Bayer and Maina, 1984; Iwuala and Okpala, 1978; Mohammed, 1977). Importantly, with special regards to bovine TBIs diagnosed to date in Nigeria, the extant literature is affected by the absence of data generated by any molecular detection tool (Leeflang and Ilemobade, 1977a,b; Obi, 1978; Akinboade and Dipeolu, 1984; Ajay and Dipeolu, 1986; Kamani *et al*., 2010).

On the whole, historically, research interests by governmental and non-governmental agencies across SSA have mainly focused on animal African trypanosomiasis (AAT) (Young *et al*., 1988). This observation may be due to the human health implications of some trypanosome species (i.e. *Trypanosoma brucei*) (Young *et al.*, 1988). This is also mirrored in the limited number of studies available to date with reference to West Africa, including Central-Northern Nigeria and the Plateau State particularly. Here, amongst other infectious diseases of their livestock, the local Fulani claim that AAT is one of the major causes of impairment of their breeding, especially during the early wet season (Awogbade, 1979). However, as described in Section 1.2, all of the several vector-borne diseases (VBDs) affecting

cattle in rural Nigeria as well as in the rest of SSA, should not be looked at individually as separate entities only because of their different vectors (e.g. tick-borne vs tsetse-borne). Conversely, a holistic approach would be desirable when aiming to understand the actual impact on animal health and productivity caused by VBDs in tropical rural Africa (de Castro, 1997; Young *et al*., 1988; Walker, 2011). Such an approach could be key to the setting up of effective control strategies, if needed (de Castro, 1997; Young *et al*., 1988; Walker, 2011).

In May 2010, a four-year research-for-development initiative entitled 'Stamp Out *Sammore*[2] (SOS)' was launched in the Plateau State, in Central Nigeria, with the aim to specifically tackle AAT in this region. Funded by the UK's Biotechnology and Biological Sciences Research Council (BBRSC) under the 'Combating Infectious Diseases in Livestock for International Development (CIDLID)' awarding scheme, the programme was carried out under the umbrella of the Nigerian Institute for Trypanosomiasis Research (NITR) in partnership with the University of Edinburgh and the French pharmaceutical multinational company CEVA Santé Animale. The project's award mainly derived from work carried out recently in the Plateau State disclosing an alarmingly high prevalence of AAT (i.e. 46.8%, 95% CI: 39.0–54.5%) (Majekodunmi *et al*., 2013). The scarcity of information on the epidemiology of cattle ticks and TBIs in the area represented both a challenge and a novelty that was integrated within the SOS programme.

[2] 'Sammore' means 'trypanosomiasis' in Hausa, the most common language in Central-Northern Nigeria.

1.2 Literature review

The literature review of this thesis will cover three subjects: ticks of importance to cattle health in SSA, the infections they can transmit to cattle and the control methods employed to date in this area. After a general overview, each section will concentrate on the tick species, TBIs and control strategies pertinent to cattle in Nigeria.

1.2.1 Ticks (Acari: Ixodida)

This review section elucidates aspects relating to the taxonomy, the biology, the distribution and the veterinary and medical importance of ticks infesting cattle in Nigeria.

1.2.1.1 Tick taxonomy

Ticks (Acari: Ixodida) are obligate haematophagous ectoparasites of vertebrates belonging to the Phylum Arthropoda (Sonenshine, 1991; Nava *et al.*, 2009). Three families are ranked within the Suborder Ixodida, namely the Ixodidae, the Argasidae and the Nuttallielidae (Guglielmone *et al.*, 2010) (Figure 1.2).

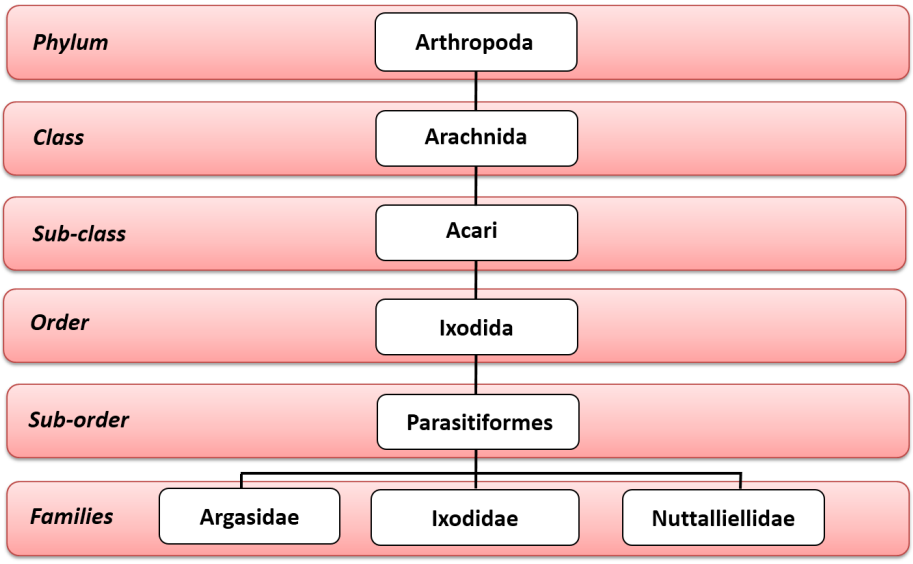

Figure 1.2 – Taxonomy of ticks [in accordance with Nava *et al.* (2009)].

The Ixodidae are also known as 'hard ticks', due to presence of a dorsal 'scutum' made of sclerotized cuticle covering their entire (in the males) or part (in the females) of their body (Sonenshine, 1991). The Argasidae are also known as 'soft ticks' due to the lack of such component. These ticks can therefore accommodate only smaller volumes of blood and can survive a longer period of starvation (Sonenshine, 1991). The family of the Nuttalliaellidae hosts only a single species (i.e. *Nuttalliela namaqua*), found in the afro-tropical region (e.g. South Africa), holding intermediate characteristics between a 'hard' and a 'soft tick' (Mans *et al*., 2014).

The family Ixodidae comprises 14 genera and 704 species and is usually grouped into Prostriata (the genus *Ixodes* only) and Metastriata (all other genera of the family). This identification is based on morphological (i.e. collocation of the anal groove compared to the anus) and biological features (Walker *et al*., 2003). The Argasidae include 5 genera and 195 species (Guglielmone *et al*., 2010).

The Ixodidae comprise the most genera and species of greatest medical and veterinary concern (Jongejan and Uilenberg, 2004). Therefore the following paragraphs elucidating aspects of biology and vector competence will mainly refer to hard ticks (Ixodidae).

1.2.1.2 Tick biology

The tick life cycle is composed of four different stages: egg, larva, nymph and adult. The adult stage is characterised by sexual dimorphism, with male and female sexes (Walker *et al*., 2003). A single gravid female can lay several hundreds to thousands of eggs, of globular shape and usually of brown colour, before dying in the environment. Oviposition lasts for several days (Sonenshine, 1991).

Depending on the tick species and the climatic conditions, eggs can hatch between two weeks to several months after laying. Each viable egg gives origin to a six-legged (i.e. hexapod) larva. Once hatched from their respective eggs, larvae start seeking a host on which they will feed for a length of time depending on each species' biological characteristics (Sonenshine, 1991).

After feeding, larvae digest their blood meal and moult into an eight-legged (i.e. octopod) nymph. Importantly, as opposed to larvae, tick nymphs and adults have

developed stigmata and tracheal systems, making them more susceptible to desiccation than the hexapod developmental stage (Needham and Teel, 1986). After moulting, nymphs will also feed for several days (i.e. 4–8) on a vertebrate host, before moulting to the next developmental stage of adults (Sonenshine, 1991).

Tick adults are sexually competent. For prostriate ticks (genus *Ixodes*) mating may occur off the host, in the environment, while for metastriates it occurs only on the host, usually simultaneously with blood feeding (Sonenshine, 1991). After mating, the engorged, fertilized female drops off the host and moves through the environment seeking a suitable site for oviposition.

According to the number of individual hosts on which they feed on throughout their life cycle, ticks can be classified as:

- **One-host ticks**: feeding and moulting on the same animal, parasitized by the larval stage of the tick. An example of one-host ticks is given by the species of the sub-genus *Boophilus* of the genus *Rhipicephalus*; their life cycle can be completed within few weeks (Walker *et al*., 2003);

- **Two-host ticks**: parasitizing two different animals throughout their cycle: one as larvae and another one as adults. Therefore larva and nymph occur on the same host. After feeding, the engorged nymph drops off the host to moult in the environment. The resulting adult would then parasitize and feed upon another host. A typical example of a two-host tick parasitizing cattle in Africa is *Rhipicephalus evertsi evertsi* (Walker *et al*., 2003);

- **Three-host ticks**: parasitizing three different animals, one for each developmental stage. All moults of three-host ticks occur in the environment. The life cycle of a three-host tick is considerably longer than that of one-host ticks, being of the order of months to years (Walker *et al*., 2003).

Moreover, some tick species (e.g. *Hyalomma* spp.) may display either a two- or a three-host life cycle, depending on environmental and climatic conditions (Sonenshine, 1991). Importantly, species of vertebrate hosts parasitized by different developmental stages of the same two- or three-host tick may differ. In particular, according to the number of vertebrate species parasitized during their life cycle, ticks can be classified also in:

- **Monotropic ticks**: feeding on the same host species throughout their life cycle. This is certainly the case of one-host ticks (e.g. *Rhipicephalus* (*Boophilus*) spp.), but also of three-host ticks such as 'the brown dog tick' *Rhipicephalus sanguineus*, feeding preferentially on dogs during all its developmental stages (Walker *et al*., 2003; Dantas-Torres, 2010);

- **Ditropic ticks**: in which larva and nymph usually parasitize a certain host species, whereas the adult feeds on another one. This is the case of *Hyalomma* species such as *Hyalomma rufipes*, parasitizing birds and cattle at the immature and adult stages respectively (Walker *et al*., 2003);

- **Telotropic ticks**: when larvae and nymph stages are able to attach on different hosts as well as the same types of hosts as the adult ticks. The 'castor bean tick' *Ixodes ricinus* displays a typical telotropic life cycle. Several other species of the genera *Dermacentor*, *Hyalomma*, *Ixodes* and *Rhipicephalus* feed on smaller vertebrates such as rodents, reptiles and even birds, as larvae and nymphs, while they feed on bigger mammals such as ungulates, carnivores and also humans as adult stages (Sonenshine, 1991).

Knowledge of these biological aspects is pivotal for the planning of effective tick control.

1.2.1.3 Veterinary and medical importance

The veterinary and medical importance of ticks is due to their blood-feeding activity, from which both their direct and indirect harmfulness take origin (Jongejan and Uilenberg, 2004). Once on a suitable host, ticks usually start blood-feeding within 24 hours (Sonenshine *et al*., 2002). Ticks' feeding seems to follow a circadian rhythm, being particularly pronounced at night (Tatchell *et al*., 1972).

1.2.1.3.1 Direct harmfulness

The direct pathogenic effects are those caused by the ticks themselves and include effects due to their blood sucking activity (e.g. blood loss leading to anaemia in the case of massive infestations; skin damage, leading to secondary bacterial infections and/or myasis) and those related to the release of components of the tick saliva characterised by inflammatory (i.e. cutaneous rush and irritation), toxic (i.e.

toxicosis) and/or sensitizing properties (i.e. allergic reactions) (reviewed in Jongejan and Uilenberg, 2004).

In order to attach to the skin of their hosts, ticks create wounds that will not entirely heal after their detachment, being rather replaced by scars (Figure 1.3). Therefore, heavy tick infestation on cattle may cause different grades of depreciation of skins and hides for the manufacture of leather, depending on the sites of attachment and the size of the mouthparts of the tick species involved (Walker, 1996; Jongejan and Uilenberg, 2004).

Figure 1.3 – Severe skin wounds on the axilla of cattle, following massive infestation by *Amblyomma variegatum* ticks.

Furthermore, ticks with particularly long mouthparts (e.g. *Amblyomma* and *Hyalomma* genera) may also induce abscess formation due to secondary bacterial infections occurring in the site of attachment (Figure 1.4), leading even to loss of teats or lameness depending on whether the body site of infestation is the udder or the interdigital clefts respectively (Walker, 1996; Ndhlovu *et al*., 2009) (Figure 1.5). Consequently, the impairment of milk secretion due to teat loss can cause an increase in calf mortality within a herd (Jongejan and Uilenberg, 2004).

Moreover, the generalized stress caused by the pruritus and the pain of tick bites, and the general annoyance of tick infestation, may also alter the normal hormonal status of lactating cows (Symons, 1985).

In addition, besides being prone to secondary infections, lesions due to tick bites can also provide entry for myiasis-causing flies (de Castro and Newson, 1993) and spread of bovine dermatophilosis by *Dermatophilus congolensis* (Actinomycetales: Dermatophilaceae), when infestation by *Amblyomma* ticks occurs (Ambrose, 1996).

(a) (b)

Figure 1.4 – (a) = *Hyalomma rufipes* adult ticks feeding on the peri-anal region of cattle. (b) = Lesions left after removal of the same ticks. Note the pus on the wounds, indicative of a secondary bacteric contamination.

Anaemia is another 'direct' outcome of the blood loss resulting from heavy infestations by any blood-sucking arthropod, including ticks (Corrier *et al*., 1979; Jongejan and Uilenberg, 2004) (Figure 1.5). With regards to ixodid ticks, most of the significant blood loss in the vertebrate host is attributable mainly to the feeding of females, whose blood meal needs to provide the amount of proteins demanded for egg-laying (Sonenshine, 1991). Inevitably, anaemia leads to loss of condition in the host, causing indirectly a reduction in weight gain (Corrier *et al*., 1979).

Figure 1.5 – Bleeding lesions in the interdigital cleft of cattle after removal of adult stages of *Hyalomma truncatum*.

Tick infestation, especially if significant, represents a cause of stress, the so-called 'tick worry' to the animals. Severely infested cattle are indeed more easily irritable, and may frequently reduce their food intake, becoming anorexic, weak and therefore more vulnerable to any other disease (Walker, 1996). Productivity losses related to direct damages have been quantified for some tick species. For instance, in the case of *Rhipicephalus* (*Boophilus*) *microplus* the main cause (i.e. 65%) of loss is seemingly represented by anorexia, due to 'tick worry', followed by blood loss (i.e. 35%; Seeback *et al.*, 1971).

Direct pathogenic effects may also result from complex interactions between the host's immune system and a wide range of antigenic proteins secreted in the tick saliva (Hart, 1994). Some of them may act as allergens or display pro-inflammatory properties causing on the feeding site hypersensitivity and inflammation respectively (Hart, 1994). This, in turn, may induce self- and mutual grooming behaviours in the host (e.g. licking, rubbing and scratching), which could potentially halt the feeding activity of ticks, by dislodging or squashing them (Allen, 1984; de Castro and Newson, 1993; Hart, 1994). Nevertheless, the accomplishment of the blood meal is

eventually ensured by a modulation of the host's immune response, due to immunosuppressive components of their saliva (Wikel and Whelen, 1986; Wikel *et al*., 1994; Schoeler and Wikel, 2001).

Furthermore, tick's saliva may contain metabolites that are toxic to the vertebrate hosts, causing several paralytic syndromes. Among the tick species present in Nigeria, this applies for *Hyalomma truncatum* and *Rh. evertsi evertsi*, known for causing the so-called 'sweating sickness' and 'lamb spring paralysis' respectively (Dolan and Newson, 1980; Viljoen *et al*., 1986).

1.2.1.3.2 Indirect harmfulness – tick-borne infections

Indirect damage to animal health due to tick infestation are mainly due the ability of these arthropods to transmit pathogens.

Ticks represent indeed a unique case among all arthropod vectors, as they may transmit the largest variety of pathogens of veterinary importance, including protozoa (e.g. *Babesia* spp. and *Theileria* spp.), rickettsiae (e.g. *Anaplasma* spp., *Ehrlichia* spp. and *Rickettsia* spp.), filarial nematodes (e.g. *Acanthocheilonema* spp. and *Cercopithifilaria* spp.) and viruses (Coltivirus, Flavivirus, Nairovirus) (Winkhardt, 1980; Olmeda-García and Rodríguez-Rodríguez, 1994; Jongejan and Uilnberg, 2004; Lasala and Holbrook, 2010).

The vectorial competence of ticks is essentially attributable to their capacity to allow, within their organs (e.g. mainly guts salivary glands, etc.) the development of infective forms of several animal and human pathogens that are then transmitted to susceptible vertebrate hosts via the tick feeding activity (Jongejan and Uilenberg, 2004). Once in the vertebrate host, these pathogens' life cycle can continue, with or without a tangible impairment of the host health (Jongejan and Uilenberg, 2004).

Ixodid ticks' blood feeding consists essentially of two phases of ingestion, more distinctively identifiable in females, due to their larger blood intake than males (Sonenshine, 1991). Briefly, a first slow phase of feeding lasting for the 6-9 days after attachment is followed by a rapid 12-24 hour long ingestion of blood, during which these ticks take two thirds of the total blood ingested, before detaching from the host (Sojka *et al*., 2013). Besides the rapid ingestion, the final phase of feeding is also characterised by a concentration of the blood nutrients, made possible via the

excretory activity of the salivary glands (Melhorn, 2008). This latter phase of the blood meal consists therefore of an alternation of rapid ingestion and rapid regurgitation of tick saliva, allowing the concentration of their blood meal, and also the excretion of salivary molecules (e.g. including antigens and allergens) and pathogens (Sonenshine, 1991).

Ticks can become infected with microorganisms through several routes of transmissions, including:

- **Systemic transmission**: the host is the source of infection ('reservoir'), acquired by the tick during the blood meal. A latency period of days or even months usually intercurs from the time the host is infected until when it can be infectious to vectors. This period is needed for the pathogen(s) to replicate in the host, reaching sufficiently high blood load enabling the vector's infection (Voordouw, 2014);

- **Co-feeding transmission** (also known as **"non-systemic transmission"**): from an infected ('donor') to an un-infected ('acquiring') tick feeding in spatiotemporal proximity to each other on the same host, in absence of a systemic infection (reviewed in Randolph *et al*., 1996). This model of transmission may be particularly important for tick-transmitted pathogens because ticks, unlike other arthropod vectors, remain attached to their hosts for several days (immature stages) or even weeks (adults) to blood feed (Randolph, 1998).

Initially described for tick-borne viruses, the 'non-systemic' model of transmission was then demonstrated also for bacteria (Randolph *et al*., 1996). In particular, two different patterns can characterize this type of transmission. Infections may in fact be acquired by originally uninfected ticks that are feeding simultaneously with infected ticks, even if there is some extent of physical separation (at least 1 cm) between them (Labuda *et al*., 1996), and this is a typical pattern of tick-borne viruses (e.g. Thogoto virus, tick-bone encephalitis virus; Louping ill flavivirus, Palma and Bhanja bunyaviruses, etc. (Jones *et al*., 1987, 1997; Labuda *et al*., 1997) Alternatively, originally uninfected ticks may acquire the infection from a localized site of the vertebrate host where infected ticks had previously fed, even after the infective

ticks have dropped off (reviewed in Randolph *et al*., 1996). The latter pattern of transmission is exhibited by *Borrelia burgdorferi*, with transmission of this spirochaete at localized skin sites of rodents being possible for several weeks from an infectious tick bite (Gern and Rais, 1996). Importantly, localized transmission may possibly occur repeatedly during the host's life even once the host is immune to the systemic infection by a certain pathogen (Randolph *et al*., 1996). For instance, sheep naturally immunized to the systemic infection by *B. burgdorferi* still permit a localized transmission of spirochaetes (Randolph *et al*., 1996).

Co-feeding in space depends on characteristics of the vertebrate hosts as well as the feeding ticks (Randolph *et al*., 1996). For instance, approximately 90% of immature (i.e. larvae and nymphs) ticks feeding on small vertebrate hosts such as rodents tend to show a marked spatial aggregation, attaching to the ears or around the eyes and on the snout, so that nearly 50% of feeding ticks is separated by less than 1 cm from each other (reviewed in Randolph, 2011). Moreover, adult stages of other tick species, such as *Rhipicephalus appendiculatus* and *A. variegatum*, feeding on larger hosts such cattle, tend to form typical clusters on the ear pinna (*Rh. appendiculatus*) (Walker *et al*., 2003) or several anatomical areas of the lower body (*A. variegatum*) (Hoogstraal, 1956; Yeoman and Walker, 1967; Walker, 1974; MacLeod, 1975; MacLeod *et al*., 1977). In fact, with regards to African TBIs of cattle, co-feeding transmission was ascertained to play a crucial role in the epidemiology of *E. ruminantium* infection (Bezuidenhout, 1987; Andrew and Norval, 1989). This is possible due to the clustering of several adult males and a fewer females on the same attachement site due to the release of the AAAP by the males (Norval and Rechav, 1979; see Section 1.2.1.5).

- **Transstadial transmission**: from a developmental stage to the next one (e.g. from larvae to nymph; from nymph to adult). When the newly infected tick stage feeds, it will pass the microorganism to its vertebrate host (Walker *et al*., 2003).

- **Transovarial transmission**: from an engorged mated female to her progenies (i.e. eggs, and therefore larvae). In this case, in fact, hatched larvae

will be infected. This route of transmission is known to occur in the case of *Babesia bovis* and *Babesia bigemina* infections (Bock *et al*., 2004);

- **Intrastadial transmission**: between ticks of the same developmental stage of the same species during co-feeding or by the same individual (i.e. male) tick feeding on several individual hosts (Potgieter, 1981; Stiller *et al.*, 1983; Zaugg *et al.*, 1986; Dagliesh *et al.*, 1987; Andrew and Norval, 1989; Norval *et al*., 1990; Kocan *et al*., 1993).

1.2.1.4 Cattle ticks in SSA

Five genera (i.e. *Amblyomma*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus*, sub-genus *Boophilus* included) of hard ticks may be found infesting cattle in SSA (Walker *et al*., 2003).

1.2.1.5 Cattle ticks in Nigeria

Three genera of ticks are usually found infesting cattle in Nigeria, namely *Amblyomma*, *Hyalomma* and *Rhipicephalus* spp. (*Boophilus* sub-genus included) (Dipeolu, 1975a; Pullan, 1980; Bayer and Maina, 1984; Walker *et al*., 2000). Infestation of the same host by several species occurs commonly, although each species' load of infestation fluctuates according to the seasons (Pullan, 1980; Bayer and Maina, 1984). Usually low in dry season (i.e. November to April), tick loads tend to increase after the first scattered rains, reaching the highest abundance one month after the heavy rains (i.e. between July and September) (Bayer and Maina, 1984; Maina, 1986). In a study carried out in Northern Nigeria over the period of one year, *Amblyomma variegatum* was the most prevalent species parasitizing cattle, followed by *Rhipicephalus* spp. (*Boophilus* spp. included) and *Hyalomma* spp. (Bayer and Maina, 1984).

1.2.1.5.1 *Amblyomma variegatum* (Fabricius, 1794)

Also known as 'the tropical bont tick', *A. variegatum* is the only species of this genus retrieved in the whole of West Africa (Petney *et al*., 1987; Walker et al., 2003). It is a medium to large tick, characterised by very long mouthparts and a typically colourful ornamentation of the scutum and conscutum (Walker *et al*., 2003) (Figure 1.6).

Figure 1.6 – Adult specimens of *Amblyomma variegatum*: male **(a)** and female **(b)**. Bar = 1mm.

As a three-host tick, *A. variegatum* spends a great part of its life cycle on the ground or vegetation before actively seeking for suitable hosts, usually cattle and small ruminants, parasitized by all three developmental stages (Petney *et al*., 1987; Walker, 1996). Nymphs can also feed on birds (Walker *et al*., 2003).

In SSA the greatest abundance of adult *A. variegatum* is generally recorded during the wet season (Petney *et al*., 1987). Previous studies report the occurrence of only one generation of *A. variegatum* per year in Nigeria, with adults being most abundant between May and June, and nymphs between December and February (Mohammed, 1976; Bayer and Maina, 1984).

Adult *A. variegatum* preferably attach to the ventral surface of the host including lower dewlap, brisket, axilla, groin and udder (Hoogstraal, 1956; Yeoman and Walker, 1967; Walker, 1974; MacLeod, 1975; MacLeod *et al*., 1977) grouping in clusters due to the presence of an 'attraction-aggregation attachment pheromone'

(AAAP), produced by fed and ready-to-mate males to attract other ticks, especially females (Norval and Rechav, 1979) (Table 1.1; Figures 1.7, 1.8).

Figure 1.7 – Infestation by *Amblyomma variegatum* forming typical clusters in the axilla of a cow.

Attaching onto the teats of cows, *A. variegatum* may interfere with suckling thus causing indirectly reduction in calf weight increase (Pegram and Chizyuka, 1990) (Figure 1.8). In this respect, studies in Cameroon (Stachurski *et al.*, 1993) and Zambia (Pegram and Oosterwijk, 1990) have shown severe calf mortality correlated to this tick infestation.

Figure 1.8 – *Amblyomma variegatum* adults on udder of cattle.

On the whole, heavy infestation by this tick species can represent a serious hazard, as its fully engorged females can weigh up to 5g each, sucking

approximately 20ml of cattle blood each (Walker, 1996). It has been estimated that, in cattle, losses of weight gain correspond to 46-61g per engorged female *A. variegatum* completing engorgement (Pegram *et al*., 1989).

Futhermore, *A. variegatum* is a competent vector of *Ehrlichia* (formerly *Cowdria*) *ruminantium*, causative agent of cowdriosis ('heartwater') (Walker and Olwage, 1987; Bezuidenhout, 1987; Deem *et al*., 1996a), *Theileria mutans* (Uilenberg *et al*., 1974; Young *et al*., 1978) and *Theileria velifera* (Uilenberg, 1981; Uilenberg and Schreuder, 1976). Moreover, *A. variegatum* is associated with dermatophilosis, a zoonotic cutaneous condition that may affect livestock (i.e. cattle, sheep, goats, horses) in West Africa and the Caribbean, caused by the commensal and mildly parasitic *D. congolensis*, whose pathogenicity can be exacerbated by the immunity suppression caused by significant infestations by the tropical bont tick (Martinez *et* al., 1993; Koney *et al.*, 1996). *A. variegatum* can also transmit the zoonotic *Rickettsia africae*, member of the spotted fever group (SFG) of rickettsiae and causative agent of African tick-bite fever (ATBF) in humans (Cazorla *et al*., 2008; Socolovschi *et al*., 2009).

1.2.1.5.2 *Hyalomma* spp.

So far, two *Hyalomma* species have been reported to infest cattle in Nigeria, namely *Hyalomma rufipes* Koch, 1844 (Figure 1.9) and *Hyalomma truncatum* Koch, 1844 (Figure 1.10) (Pullan, 1980; Bayer and Maina, 1984). Like *Amblyomma* spp., *Hyalomma* ticks are medium-sized to large ticks with typically long mouthparts, preferably feeding on large herbivores (i.e. cattle, sheep, goat, horses, camels, etc.) at the adult stage, but also on domestic dogs in the case of *H. truncatum* (Walker *et al*., 2003). Immature stages of *H. rufipes* usually attach on hares and ground-frequenting birds; those of *H. truncatum* feed on hares and rodents, especially gerbils, and can also parasitize humans (Walker *et al*., 2003).

Both *H. rufipes* and *H. truncatum* can modulate their development according to the host and perhaps other ecological factors, exhibiting either a two- or three-host life cycle (Magano *et al*., 2000). Adults of both species are most numerous during

the late wet season, whereas the immature stages are most prevalent in the dry months (Walker *et al*., 2003).

Figure 1.9 – Adult specimens of *Hyalomma rufipes*: male **(a)** and female **(b)**. Bar = 1 mm.

Figure 1.10 – Adult specimens of *Hyalomma truncatum*: male **(a)** and female **(b)**. Bar = 1 mm.

Hyalomma rufipes is known to transmit *Anaplasma marginale* (Potgieter, 1979), *Babesia occultans* (Blouin and van Rensburg, 1988) and *Theileria annulata* (Jongejan *et al*., 1983) to cattle.

Although it does not serve as a vector of haemoparasites, *H. truncatum* does still represent a hazardous species, mainly due to the preferential localization of its adult stages on the interdigital clefts, which may lead to lameness in cases of heavy infestation (Kok and Fourie, 1995) (Table 1.1). In addition, some strains of *H. truncatum* are also known for causing the so-called 'sweating sickness', an acute toxicosis particularly severe in young cattle, characterised by moist eczema in the infested body parts (van Amstel *et al.*, 1987a).

Moreover, both *H. rufipes* and *H. truncatum* are involved in the transmission of the Crimean-Congo haemorragic fever virus and other viruses to humans (Shepherd *et al*., 1989).

1.2.1.5.3 *Rhipicephalus* (*Boophilus*) spp.

Those belonging to the *Boophilus* sub-genus are small one-host ticks developing throughout their entire life cycle (i.e. from unfed larva to fed adult stage) on the same animal (Walker *et al*., 2003).

On average, boophilid ticks can complete their life cycle in approximately 80 days (i.e. three weeks feeding on host and two months from egg laying to larval development), having up to five generations per year in presence of optimal equatorial environments (Walker *et al*., 2003; Walker, 2011).

Although their mouthparts are short, 'boophilid' ticks can still cause considerable skin damage, with consequent depreciation of hides, by attaching in large number preferentially to body areas of good leather potential such as flanks, dewlap and dorsum (Jongejan and Uilenberg, 2004; see Table 1.1; Figure 1.9).

Figure 1.9 – Heavy infestation by *Boophilus* spp. on the low dewlap of a White Fulani cattle.

Besides the direct damages, the veterinary importance of *Boophilus* ticks is mainly associated to their capacity to act as vectors of *Anaplasma* spp. and *Babesia* spp. (Rees, 1950; Akinboade and Dipeolu, 1983; Büscher, 1988; Samish *et al.*, 1993; Hodgson, 2006).

With regards to Nigeria, thus far three *Boophilus* species have been found infesting cattle, namely *Rhipicephalus* (*Boophilus*) *decoloratus* (Koch, 1844) (Figure 1.12), *Rhipicephalus* (*Boophilus*) *annulatus* (Say, 1981) and *Rhipicephalus* (*Boophilus*) *geigyi* (Aeschliman and Morel, 1965) (Dipeolu, 1975a).

For all three species, cattle are considered as the host species responsible for the maintenance of their population in given geographic area (Walker *et al*., 2003). Nevertheless, these boophilids may also succesfully accomplish their life cycles on small ruminants and wild ungulates (Walker *et al*., 2003).

Usually known as 'the blue tick' or 'the Africa blue tick' due to the chromatographic features of the cuticle of its engorged females, *Rh.* (*Bo.*) *decoloratus* is known as the most widespread tick species in Nigeria (Dipeolu, 1975a) (Figure 1.12), followed by *Rh.* (*Bo.*) *annulatus*, known as the most common cattle tick in eastern Nigeria (Imwuala and Okpala, 1978).

Both *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *annulatus* are known as vectors of *Anaplasma marginale*, *Anaplasma centrale* and *Babesia bigemina* (Akinboade, 1981; Adetunji *et al*., 1981; Akinboade, 1981; Akinboade and Dipeolu, 1983; Büscher, 1988; Samish *et al.*, 1993; Bock *et al*., 2004). Moreover, both tick species

can also transmit *Borrelia theileri* (Laveran 1903) Bergey *et al*. 1925 (Neitz, 1956, Trees, 1978), a spirochete of low clinical importance (Smith *et al*., 1985), probably widespread among Nigerian cattle (Trees, 1978).

Figure 1.10 – Adult specimens of *Rhipicephalus* (*Boophilus*) *decoloratus*: male **(a)** and female **(b)**. Bar = 1 mm. Note the rather small size of these ticks.

Due to similar morphological features, *Rh.* (*Bo.*) *geigyi* is often misidentified with *Rh.* (*Bo*.) *decoloratus* (Walker *et al*., 2003). The distribution of the former tick species, however, is restricted to the warmer and more humid areas of West Africa, being more abundant during the dry months (Estrada-Peña *et al*., 2006). Although it is suspected to be involved in the transmission of *Babesia* spp. piroplasms to cattle (i.e. *B. bovis*) (Dipeolu, 1975b; Akinboade and Akinboade, 1985), thus far the vectorial role of *Rh.* (*Bo.*) *geigyi* has been scantly investigated.

1.2.1.5.4 The threatening spread of *Rhipicephalus* (*Boophilus*) *microplus*

Importantly, over the past few years, cattle rearing in Nigeria as well as in much of West Africa, has been jeopardized by the rapid expansion of another boophilid tick species. Also known as 'the pantropical blue tick' or 'the cattle tick' or 'the Asian cattle tick', *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1888) probably originated in Asia, but since the second half of the nineteenth century it has been spread worldwide via the trading of cattle, establishing in several regions due to its adaptability and highly invasive behaviour (reviewed in Estrada-Peña *et al*., 2006). As for the three aforementioned boophilid species, also for *Rh.* (*Bo.*) *microplus* cattle are considered the maintenance host (Walker *et al*., 2003).

At present, *Rh.* (*Bo.*) *microplus* is widespread in Latin America, Asia, Australia, Southern Africa and Madagascar (Kumar *et al*., 2002; Estrada-Peña *et al*., 2006; Islam *et al*., 2006; Yamane *et al*., 2006; Durden *et al*., 2008; Changbunjong *et al*., 2009; Cotullé *et al*., 2009; Chen *et al*., 2010). Before 2007, the distribution of this tick in SSA was limited to Southern Africa (i.e. Mozambique, South Africa, Swaziland, Zambia and Zimbabwe) (Howell *et al*., 1978; Mason and Norval, 1980; Berkvens *et al*., 1998; Weddeburn *et al*., 1999; Horak *et al*., 2009), where it was initially introduced from cattle from Madagascar, originally imported from South-East Asia (Barré and Uilenberg, 2010).

In 2007, however, *Rh.* (*Bo.*) *microplus* was recorded for the first time in the Ivory Coast, where it was likely introduced through cattle imported from Brazil (Madder *et al*., 2007). Since then, this tick's geographical range has expanded rapidly, being recorded by now in four other West African countries including Benin (Madder *et al*., 2011, 2012; De Clercq *et al*., 2012), Burkina Faso, Mali and Togo (Adakal *et al*., 2013) (Figure 1.13). Moreover, very recently, *Rh.* (*Bo.*) *microplus* was also reported also in cattle from Namibia, though its introduction in this country most likely happened via South Africa (Nyangiwe *et al*., 2013).

The veterinary public health relevance of this tick species gaining ground in SSA lies on its ascertained role as a competent vector of the highly pathogenic *B. bovis* (Potgieter and Els, 1976) as well as of *B. bigemina* (Riek, 1964), *A. marginale*

(Futse *et al.*, 2003) and its increasingly diffuse acaricide resistance (Baffi *et al.*, 2008).

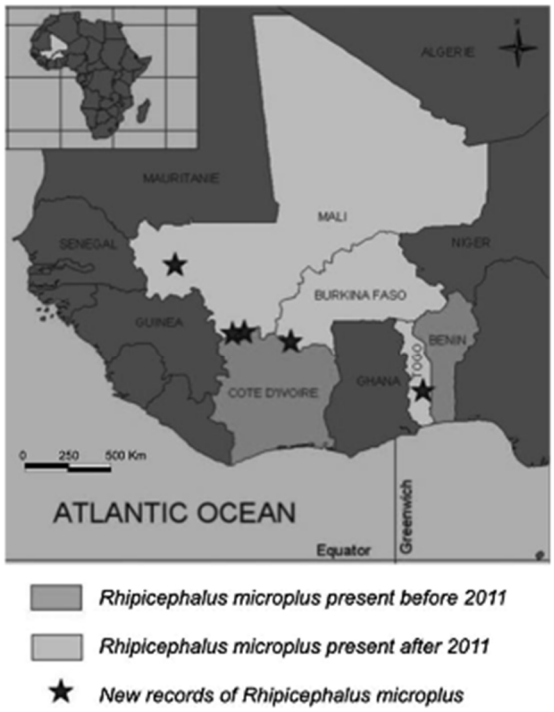

Figure 1.11 – *Rhipicephalus* (*Boophilus*) *microplus* in West Africa: up-to-date distribution (from Adakal *et al.*, 2013).

1.2.1.5.5 *Rhipicephalus* spp.

Besides the *Boophilus* sub-genus, the *Rhipicephalus* genus includes the highest variety of species that could be retrieved on cattle in several parts of Nigeria. These include *Rh. evertsi evertsi*, *Rhipicephalus guilhoni* (Figure 1.12), *Rhipicephalus lunulatus*, *Rhipicephalus muhsamae*, *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* (Bayer and Maina, 1984; Okaeme, 1986; Walker *et al.*, 2000; Ogo *et al.*, 2012). Among them, *Rh. evertsi evertsi* (Neumann, 1897) is the most hazardous to cattle health as it may be involved in the transmission of *A. marginale* (Gueye *et al.*, 1994).

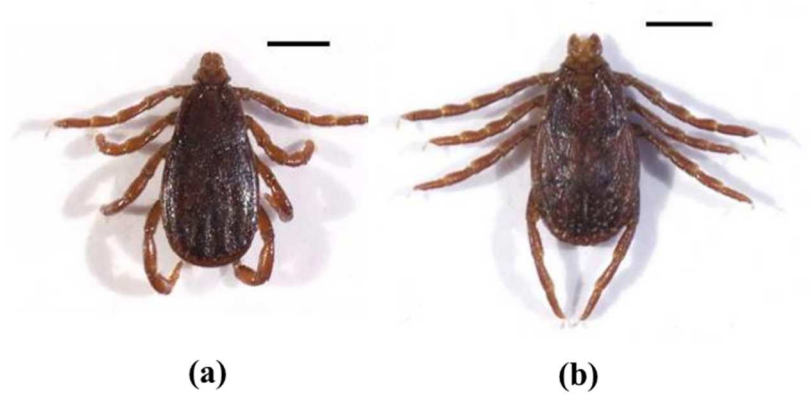

Figure 1.12 – Adult specimens of *Rhipicephalus guilhoni*: male **(a)** and female **(b)**. Bar = 1 mm.

With a two-host life cycle, this species is also known as 'the red-legged tick' due to the bright orange colour of its legs. Adult stages preferentially feed on cattle, sheep, horses and donkeys, where also larvae and nymphs can be found (Walker *et al*., 2003). Immature stages can also infest hares and various small antelope species (Walker *et al*., 2003). Moreover, a wide variety of wild ungulates can be infested by all developmental stages of *Rh. evertsi evertsi* (Walker *et al*., 2003).

On the cattle host, adult specimens are commonly found on the hairless area around the anus (see Table 1.1, Figure 1.13), whereas the immature stages attach to the inner surface of the ear (Walker *et al*., 2003).

Figure 1.13 – *Rhipicephalus evertsi evertsi* adults typically localized in the peri-anal region of cattle.

Females of *Rh. evertsi evertsi* are also known for being able to release toxins causing paralysis to sheep, goat and cattle (Gothe and Bezuidenhout, 1986).

Rh. sanguineus (Latreille, 1806), commonly known as 'the brown dog tick', is the most widespread tick species in the tropics and sub-tropics, of great canine health concern (Dantas-Torres, 2008). It is a small brownish-red three-host tick that may occasionally be found feeding on cattle that come in contact with dogs (preferential hosts) or grazed in the vicinity of dogs' premises (Walker *et al*., 2003). With reference to bovine pathogens, an Australian population of this tick proved able to transmit *A. marginale* transstadially under experimental conditions (Parker, 1982), though up to now this vectorial competence has not been further demonstrated in the field.

Importantly, several *Rhipicephalus* spp. (e.g. *Rh. sanguineus* Group) can also transmit zoonotic rickettsiae causative agents of 'spotted fevers' in humans (reviewed in Cazorla *et al*., 2008).

Table 1.1 – Predilection sites of attachment of tick species known to parasitize cattle in Nigeria.

Tick species	Preferential attachment sites	Reference
Amblyomma variegatum	Low dewlap, brisket, axillae, groin (i.e. udder or scrotum)	Hoogstraal (1956) Yeoman and Walker (1967) Walker (1974) MacLeod (1975) MacLeod *et al*. (1977)
Hyalomma Rufipes	Peri-anus, abdomen, groin	MacLeod (1975)
Hyalomma truncatum	Peri-anus, tail base, abdomen, groin, interdigital cleft	MacLeod (1975)
Rhipicephalus (*Boophilus*) spp.	Dewlap, flanks	MacLeod (1975) Walker *et al*. (2003)
Rhipicephalus evertsi evertsi	Immature stages: Ear canal Adult stages: Peri-anus	MacLeod (1975)

Tick species	Preferential attachment sites	Reference
Rhipicephalus guilhoni	Ears	MacLeod (1975)
Rhipicephalus sanguineus	Ears	MacLeod (1975)

1.2.2 Cattle tick-borne infections (TBIs) in SSA

Amongst others, the most important pathogens transmitted by hard ticks to cattle in SSA belong to the genera of protozoa *Babesia* (Piroplasmida: Babesiidae) and *Theileria* (Piroplasmida: Theileriidae) and of rickettsial microorganisms *Anaplasma* (Rickettsiales: Anaplasmataceae) and *Ehrlichia* (Rickettsiales: Anaplasmataceae) (reviewed in Minjauw and McLeod, 2003; Jongejan and Uilenberg, 2004). Besides their role as 'competent' vectors directly involved in the life cycle of the pathogens they transmit, allowing their development into infective stages, ticks may also be implicated in the mechanical transmission of hazardous microorganisms, as occurs for *Amblyomma variegatum* and the bacterium *D. congolensis* (Actinomycetales: Dermatophilaceae) (Koney *et al*., 1996).

1.2.2.1 Cattle TBIs in Nigeria

In the first half of the twentieth century, TBIs in Nigeria were regarded more as pathological syndromes occurring in conjunction with other conditions (e.g. rinderpest, trypanosomiasis) instead of being independently implicated in the outbreak of diseases (Leeflang and Ilemobade, 1977a). Later on, the existence of diseases exclusively caused by tick-borne pathogens became progressively more tangible, especially with the establishment of delimited zones for livestock rearing such as grazing reserves, stock farms etc. (Leeflang and Ilemobade, 1977a). This was probably not only attributable to the concentration of animals within confined zones, but also to an increase of awareness within the veterinary sector (Leeflang and Ilemobade, 1977a).

Due to the diffuse distribution of their competent vectors, four bovine tick-borne infections are widespread in Nigeria, namely anaplasmosis, babesiosis, cowdriosis and theileriosis (Dipeolu, 1975b; Leeflang and Ilemobade, 1977a,b). They are usually characterised by two clinically apparent presentations: an acute and a per-acute form (see Table 1.3). Sub-clinical/chronic forms are the least likely identifiable clinically, being also the most likely to occur in indigenous (*B. indicus*) cattle in rural SSA.

1.2.2.1.1 Anaplasmosis ('gall sickness')

In Nigeria, bovine anaplasmosis, also known as 'gall sickness', is caused by the rickettsial microorganisms *A. marginale* Theiler, 1910 and *A. centrale* Ristic and Kreier 1984 (Rickettsiales: Anaplasmataceae) (Leeflang and Ilemobade, 1977a,b; Obi, 1978; Akinboade and Dipeolu, 1984; Ajayi *et al*., 1987; Egbe-Nwiyi *et al*., 1997), though the latter species has never been detected in Northern Nigeria (Leeflang and Ilemobade, 1977b).

While *A. marginale* is known for being more pathogenic, *A. centrale* is usually associated with a sub-clinical form of the disease (Minjauw and McLeod, 2003; reviewed in Aubry and Geale, 2011).

Once inoculated in the vertebrate host by their tick vectors, *A. marginale* and *A. centrale* invade the erythrocytes, in which they replicate without causing cell lysis (Aubry and Geale, 2011). Interestingly, the different denomination given to these two species is attributable to their typical localization within the erythrocyte cytoplasm: peripheral (or 'marginal') for *A. marginale* (Theiler, 1910) and rather centralized for *A. centrale* (Dumler *et al.*, 2005).

Considering that, in Nigeria, both *A. marginale* and *A. centrale* share, amongst others, vector ticks that can also transmit *B. bigemina* (Adetunji *et al*., 1981; Akinboade and Dipeolu, 1983; Samish *et al.*, 1993), mixed infections by *Anaplasma* and *Babesia* spp. should be considered as very likely to occur (Ajayi and Dipeolu, 1986; see Table 1.2).

In addition, biting flies (*Stomoxys* spp.), horse flies (*Chrysops* spp., *Tabanus* spp.) and horn flies (*Haematobia* spp.), as well as blood-contaminated fomites may

also act as mechanical vectors of *Anaplasma* spp. (Foil, 1989; Scoles *et al*., 2005; Aubry and Geale, 2011; Baldacchino *et al*., 2013).

Generally the main clinical signs of anaplasmosis are fever (39.4–41.7°C), anaemia (Ajayi *et al*., 1987), which can be particularly severe at the peak of rickettsiaemia when 10–30% of erytrhocytes are infected (Young *et al*., 1988), icterus, anorexia and interruption of rumination with consequent constipation and weakness (Richey and Palmer, 1990; Potgieter and Stoltz, 1994; Egbe-Nwiyi *et al*., 1997) (Table 1.3). However, acute anaplasmosis mainly affects exotic breeds moved into endemic areas, or local adult cattle bred under conditions of poor nutrition (Richey and Palmer, 1990) or affected by other concurrent infections (Aubry and Geale, 2011).

Most of the losses due to anaplasmosis include reduction in weight gain and milk yields, abortion, mortality up to 50% and cost of veterinary treatments (Kocan *et al*., 2003).

Cattle that recover from sub-clinical or acute anaplasmosis develop a solid lifelong immunity against the apparent form of the disease (Richey and Palmer, 1990). Nonetheless, they still play a crucial role in the epidemiology of anaplasmosis as they remain 'carriers' of its causative agent, being persistently infected with repetitive cycles of rickettsiaemia and thus serving as reservoirs of infection for feeding boophilid ticks that may transmit the infection to other susceptible vertebrate hosts (Eriks *et al*., 1989).

Bovine anaplasmosis can be treated with oxytetracyclines and imidocarb proprionate (Aubry and Geale, 2011).

1.2.2.1.2 *Anaplasma bovis* Dumler *et al*., 2001

Another tick-borne haemoparasite member of the family Anaplasmataceae that may be found infecting cattle in Nigeria is *Anaplasma bovis* Dumler *et al*., 2001 (Leeflang and Ilemobade, 1977a), parasitizing monocytes in the bloodstream, causing a benign form of bovine ehrlichiosis (Donatien and Lestoquard, 1936).

Its distribution was thought to be limited to countries of the Mediterranean basin until the 1960s (Bertram, 1962), thereafter its presence started being

documented also in SSA, where it was demonstrated to be transmitted by ticks of the genus *Hyalomma* (Neitz, 1956).

The first, and to date only, documented cases of *A. bovis* infection in Nigerian cattle, report of an unusual localization of this rickettsia in the cytoplasm of lymphocytes rather than monocytes (Leeflang and Ilemobade, 1977a).

1.2.2.1.3 Babesiosis ('redwater fever')

In Nigeria, so far cases of bovine babesiosis have been attributed to two species of piroplasms: the widespread *B. bigemina* (Angus, 1996) (Leeflang and Ilemobade, 1977b; Ajayi *et al*., 1982; Akinboade and Dipeolu, 1984; Akinboade and Akinboade, 1985; Ajayi and Dipeolu, 1986) but also the highly pathogenic *B. bovis* (Angus, 1996) (Leeflang and Ilemobade, 1977b; Akinboade and Dipeolu, 1984; Ajayi and Dipeolu, 1986; Kamani *et al*., 2010) (Table 1.2).

Babesia spp. (Eucoccidiorida: Babesiidae) parasitize the erythrocytes of their vertebrate hosts, in which they replicate by binary fission (i.e. merogony), at the end of which the newly formed merozoites cause the red blood cell to lyse (reviewed in Chauvin *et al*., 2009).

While the former species has been demonstrated to be vectored by *Rh.* (*Bo.*) *decoloratus* in Nigeria (Leeflang and Ilemobade, 1977b; Adetunji *et al*., 1981; Akinboade, 1981; Akinboade *et al.*, 1981; Akinboade and Dipeolu, 1983) and could potentially be vectored also by *Rh.* (*Bo.*) *annulatus* (Bock *et al.*, 2004), the competent vector of *B. bovis* remains yet to be clarified in this country.

The first written report of the presence of *B. bigemina* in Nigeria was documented in 1914 in cattle presented to a slaughterhouse (reviewed in Leeflang and Ilemobade, 1977a). Already in the early 1920s, babesiosis was known to be endemic in most regions of Nigeria (Leeflang and Ilemobade, 1977a). At the time, increased mortality attributed to babesiosis was reported in young stock as a consequence of rinderpest immunization, temporarily challenging the immune system of calves, thus favouring the onset of clinical piroplasmosis (Leeflang and Ilemobade, 1977a). In the following decades cases of babesiosis used to be diagnosed in conjuction with trypanosomiasis (e.g. *Trypanosoma congolense*) (Leeflang and Ilemobade, 1977a; Ajayi, 1978).

The first infections distinctively caused by *B. bovis* were diagnosed in 1956 just in the Plateau province, when the detection of this parasite was mentioned in two reports (Leeflang and Ilemobade, 1977a). Afterwards, a few other reports identified this pathogenic babesia in Nigerian cattle by means of cytological examination of blood smears (Akinboade and Dipeolu, 1984; Kamani *et al.*, 2010) and indirect fluorescent antibody test (IFAT) (Ajayi and Dipeolu, 1986).

As with anaplasmosis, babesiosis also tends to be more severe in non-resistant exotic (*B. taurus*) cattle, though indigenous breeds can be greatly clinically affected, especially by *B. bovis* infections, in the presence of other concomitant infections and/or poor nutrition (Ajayi, 1978).

Clinical signs in infections with *B. bigemina* and *B. bovis* are similar. Commonly known as 'redwater fever' or simply 'redwater', bovine babesiosis is clinically characterised initially by fever (41–42˚C), which can persist for a week or more, followed by haemolytic anemia leading to haemoglobinuria (explaining the common name of the disease), inappetence and weakness (Hildebrandt, 1981; see Table 1.3).

The predominant clinical symptoms are, however, anaemia and haematuria (Young *et al.*, 1988; Jonsson *et al.*, 2008) (Table 1.3). The latter sign is pathognomonic, considering that it can enable the differentiation of babesiosis from anaplasmosis and cowdriosis (Young *et al.*, 1988).

As for cowdriosis, the per-acute form of babesiosis by *B. bovis* may be characterised by neurological signs (e.g. ataxia, paddling of limbs, incoordination, convulsions and even coma), leading the animal to death within a week after the onset of clinical signs (Hildebrandt, 1981).

Animals that recover from acute babesiosis become carriers for a number of years with *B. bovis* or for a few months in the case of *B. bigemina* (Zintl *et al.*, 2005). However they can still develop the disease again if subjected to stress or any other cause of debilitation (e.g. malnutrition, concurrent infections, hormonal changes, etc.) (Jonsson *et al.*, 2008).

Currently diminazene aceturate and imidocarb diprionate are the most widely employed babesicidal drugs (de Vos and Potgieter, 1994). Diminazene protects cattle against *B. bovis* and *B. bigemina* for two and four weeks respectively (de Vos, 1979),

while imidocarb provides protection for 4 weeks for *B. bovis* and for at least two months for *B. bigemina* (Taylor and McHardy, 1979).

1.2.2.1.4 Cowdriosis ('heartwater')

Cowdriosis is a condition of ruminants caused by the rickettsial microorganism *Ehrlichia* (formerly *Cowdria*) *ruminantium* (Cowdry, 1926) (Rickettsiales: Anaplasmataceae), transmitted by *A. variegatum* (Walker and Olwage, 1987; Leeflang and Ilemobade, 1977b), whose preferential host is represented by cattle (Petney *et al*., 1987) (Table 1.2).

Bovine cowdriosis is considered to be the most important TBD in Nigeria (Ilebomade, 1976; Ilemobade and Leeflang, 1977; Leeflang and Ilemobade, 1977a) and second only to East Coast Fever caused by *Theileria parva* (Theiler, 1904) in the whole of SSA (Uilenberg, 1983).

Within its tick vector, *E. ruminantium* is transmitted transstadially (Bezuidenhout, 1987), with transovarial transmission having been demonstrated for *Amblyomma hebraeum* only under experimental conditions (Bezuidenhout and Jacobsz, 1986) and not likely to occur in the field for *A. variegatum* (Kocan, 1995).

In the vertebrate host, *E. ruminantium* proliferates in the vascular endothelial cells, neutrophils, macrophages and reticulo-endothelial cells (Prozesky and Du Plessis, 1987).

The first described case of cowdriosis in Nigeria was documented by cytological examination of preparations from several tissues of sheep at Vom, in the Plateau, in 1928 (Leeflang and Ilemobade, 1977a). A few years later, the occurrence of the disease was confirmed using the diagnostic approach proposed by the Veterinary Research Laboratory in Pretoria, South Africa, according to which the presence of rickettsiae had to be disclosed in smears from the intima of the jugular vein and posterior vena cava (Leeflang and Ilemobade, 1977a). Soon after, it became clear that this TBI was widespread among cattle and sheep in the country, before being diagnosed for the first time also in goats in 1956 (Leeflang and Ilemobade, 1977a).

Because of the seasonality of its tick vector, developing one generation per year in Nigeria (Mohammed, 1974, 1977; Bayer and Maina, 1984), cowdriosis is

usually expected to occur during the early rainy season (i.e. May to August), corresponding to the period of maximal activity of adult females (Bayer and Maina, 1984) and, to a minor extent in the midst of the dry season (i.e. December to February), when nymphal stages abound (Mohammed, 1974, 1977).

Its common name 'heartwater' is derived from the typical hydropericardium often seen at *post-mortem* examination, mainly due to the increased vascular permeability caused by the microorganism, which proliferates within the endothelial cells of blood vessels (Uilenberg and Camus, 1993). The neurological signs, in fact, are due to the particular tropism of *E. ruminantium* for the endothelium of the brain's blood vessels (Prozesky, 1987). Other main clinical signs include fever, anorexia, listlessness and diarrhoea (van Amstel *et al.*, 1987b; see Table 1.3). Cowdriosis should be taken into account in the differential diagnosis with babesiosis, as it can also exhibit neurological signs in per-acute form (Uilenberg and Camus, 1993) (Table 1.3).

Similarly to anaplasmosis and babesiosis, post-acute *E. ruminantium* infections are characterised by durable chronic infections in asymptomatic carrier animals (Ilemobade, 1978; Andrew and Norval, 1989), which serve as reservoirs of the infection for *A. variegatum* ticks (Deem *et al.*, 1996a).

In Nigeria, heartwater is usually treated with two doses of oxytetracycline given at a 48 hour-interval (Leeflang and Ilemobade, 1977b; Maina, 1986). Importantly, it was noticed that the treatment had less chance to be effective if the first oxytetracycline administration was given later than two days from the onset of pyrexia (Leeflang and Ilemobade, 1977b).

1.2.2.1.5 Theileriosis

To date, two theilerial species are known to be found infecting cattle in Nigeria: *Theileria mutans* (Theiler, 1906) and *Theileria velifera* (Uilenberg, 1964). In the bloodstream of livestock they can be detected in lymphocytes and erythrocytes where different parts of their life cycle (i.e. schizogony and merogony, respectively) take place (Norval *et al.*, 1992; Bishop *et al.*, 2004).

1.2.2.1.5.1 *Theileria mutans* (Theiler, 1906)

Like *B. bigemina*, *Theileria* (Piroplasmida: Theileriidae) microorganisms were first described in the blood of Nigerian cattle in 1914 in a slaughterhouse (reviewed in Leeflang and Ilemobade, 1977a). By 1930, it was thought that theileriosis alone or in concomitance with *B. bigemina*, could cause illthrift in calves (Leeflang and Ilemobade, 1977a). In the early 1980s theileriosis was known as the most prevalent TBI of cattle in Zaria province, in Central-Northern Nigeria, where prevalence, detected by means of cytology, reached up to over 60% (Saidu *et al*., 1984). Other studies suggested the presence of this piroplasm to be widespread in the country, with no apparent difference between traditional and intensive farming systems (Dipeolu, 1975b; Saidu *et al*., 1984).

Thought to have evolved in the African or Cape buffalo (*Syncerus caffer*) (Uilenberg, 1981), *T. mutans* is infective for cattle and it may seemingly cause latent infections in sheep (Paling *et al*., 1981). *T. mutans* is known to be of low pathogenicity to cattle (Norval *et al*., 1992), though it is believed that buffalo-derived stocks are more pathogenic than the cattle-derived ones (Young *et al*., 1978).

T. mutans can be transmitted by at least five *Amblyomma* ticks such as *Amblyomma cohaerens* (Young *et al*., 1977a), *Amblyomma gemma* (Paling *et al*., 1981), *A. hebraeum* (de Vos and Roos, 1981; Lawrence *et al*., 1981), *Amblyomma lepidum* (Morzaria *et al*., 1981) and *A. variegatum* (Uilenberg *et al*., 1974; Young *et al*., 1978), of which only the latter species can be found in Nigeria (Walker *et al*., 2003) (Table 1.2).

Anaemia is the main clinical sign; possibly accompanied by jaundice, with rare episodes of haematuria (Young *et al*., 1978; Uilenberg, 1981).

The pathogenicity of this piroplasm is indeed related to the grade of its parasitaemia; which in most cases is low (up to four infected erythrocytes per 1,000) (Saidu, 1981) but durable, causing a prolonged, nearly life long, carrier state (Norval *et al*., 1992).

Nevertheless, under certain circumstances, *T. mutans* may become responsible for disease, primarily as a result of anaemia induced by the high levels of intra-erythrocytic merogony of this parasite, likely to lead to red blood cell lysis, especially in mixed infections with other pathogens (Bishop *et al.*, 2009). Fatal

infections have indeed been reported in East Africa (Irvin *et al*., 1972). Moreover, in Uganda, it was suggested that *T. mutans* might induce clinical theileriosis in cattle, in apparent absence of concurrent infections by the more pathogenic *Theileria parva* (Oura *et al*., 2004). Furthermore, immunosuppresion due to concurrent trypanosome infections may also promote theileriosis by these benign *Theileria* species (Bishop *et al*., 2009). In Nigeria, early reports suggested the possible implication of *T. mutans* in cases of illness, though it was ultimately assumed that 'a certain degree of stress' was needed for a condition to become manifest (Saidu, 1981). To date, however, mortality has never been associated with the infection in this country (Leeflang and Ilemobade, 1977a,b).

1.2.2.1.5.2 *Theileria velifera* (Uilenberg, 1964)

Theileria velifera is the other theilerial species known to occur in cattle in Nigeria (Perié *et al*., 1979; Saidu *et al*., 1984). Similar to *T. mutans*, this species is also believed to be originally a parasite of Cape buffalo (Uilenberg, 1981). It is of low pathogenicity and in Nigeria shares the same competent vector with *T. mutans*, namely *A. variegatum* (Uilenberg, 1981; Uilenberg and Schreuder, 1976) (Table 1.2).

Table 1.2 – Bovine TBIs in Nigeria.

Tick species names within square brackets are indicative of vector compotences yet to be confirmed for Nigeria.

Infection / Disease	Common Name	Causing microorganism(s)	Tick vector	Reference
Anaplasmosis	Gall sickness	*Anaplasma marginale*	- *Rh.* (*Bo.*) *annulatus* - *Rh.* (*Bo.*) *decoloratus* - *Rh. evertsi evertsi* - *Rh. simus*	Potgieter (1981); Samish *et al*., (1993); Gueye *et al*. (1994)
		Anaplasma centrale	- *Rh.* (*Bo.*) *annulatus* - *Rh.* (*Bo.*) *decoloratus*	Potgieter (1981); Shkap *et al*. (2009)
		Anaplasma bovis	- *Hyalomma* spp.	Neitz (1956)
Babesiosis	Redwater (fever)	*Babesia bigemina*	- *Rh.* (*Bo.*) *decoloratus* - *Rh.* (*Bo.*) *annulatus*	Adetunji *et al*. (1981); Akinboade (1981)
		Babesia Bovis	- [*Rh.* (*Bo.*) *geigyi*] - [*Rh.* (*Bo.*) *annulatus*]	Akinboade and Dipeolu (1981, 1983); Büscher (1988)
Cowdriosis	Heartwater	*Ehrlichia Ruminantium*	- *Amblyomma variegatum*	Walker and Olwage (1987); Bezuidenhout (1987)
Theileriosis		*Theileria mutans* *Theileria velifera*	- *Amblyomma variegatum*	Uilenberg *et al*. (1974); Young *et al*. (1978)

Table 1.3 – Main clinical signs and production losses associated to the predominant vector-borne diseases (VBDs) of cattle in Nigeria.
Trypanosomiasis is included in the list to give the reader a holistic view on possible presentations occurring in the field due to co-infections by multiple VBDs.

Disease	Possible clinical signs	Production losses	Reference
Anaplasmosis	• Per-acute form: - Death within a few hours from the onset of the same clinical signs of the acute form. • Acute form: - Fever (39.4–41.7°C for 3-7 days); severe anaemia; icterus; interruption of rumination; anorexia; constipation; weakness; dyspnoea.	- Weight loss; - Reduction in milk and meat yields; - Abortion; - Mortality up to 50%.	Ajayi *et al*. (1978); Richey and Palmer (1990); Potgieter and Stoltz (1994); Egbe-Nwiyi *et al*. (1997); Jonsson *et al*. (2008)
Babesiosis	• Per-acute form (usually caused by *B. bovis*): - Progressive neurological signs [lethargy, ataxia, incoordination, hyperesthesia, convulsions] + same signs of the acute form. - rapid death. • Acute form: - Fever (~41°C or higher); lethargy; anaemia; haemoglobinuria; icterus; diarrhoea.	- Weight loss; - Mortality; - Reduction in milk and meat yields; - Deterioration of internal organs (e.g. liver, spleen).	Ajayi (1978); Hildebrandt (1981); Akinboade and Akinboade (1985); Jonsson *et al*., (2008)
Cowdriosis	• Per-acute form: - High fever (> 41°C); dyspnea; hyperesthesia; lacrimation; occasional diarrhoea; - rapid death. • Acute form: - Sudden fever; anorexia; diarrhoea; dyspnoea and coughing; hydrothorax; - progressive neurological signs [i.e. Early stage: Chewing movements, protrusion of the tongue, twitching of the eyelids, circling, often with a high-stepping gait, muscle rigidity with tremors, behavioural changes. Intermediate stage: Convulsions. Late stage:	- Weight loss; - Mortality; - Reduction in milk and meat yields; - Depreciation of carcasses; - Deterioration of internal organs.	Ilemobade (1976); Uilenberg (1983); Prozesky (1987); van Amstel *et al.*, (1987b)

Disease	Possible clinical signs	Production losses	Reference
	Permanent lateral recumbency with paddling or galloping movements, opisthotonos, hyperesthesia, nystagmus and frothing at the mouth] - Death within a week from onset of clinical signs. • Sub-acute / Chronic form: - Prolonged fever; coughing; mild incoordination.		
Trypanosomiasis	• Acute form: - Swelling at the site of the fly bite; intermittent fever; anaemia; lymphadenopathy; anorexia; emaciation; diarrhoea; lacrimation; keratitis; - death within a week from the onset of clinical signs. • Chronic form: - Attenuated form of clinical signs of the acute disease.	- Weight loss; - Reduction in milk and meat yields; - Mortality; - Abortions; - Stillbirths; - Infertility.	Ikede *et al*. (1988); Murray and Dexter (1988); Clausen *et al*., (1993)

1.2.3 Endemic (enzootic) stability

When dealing with TBIs and indigenous (*B. indicus*) cattle in rural SSA, the existence of endemic stability should always be considered.

Endemic (or 'enzootic', as it refers to animals, in this case) stability refers to the epidemiological state characterised by the absence of clinical disease within a certain host population, in the presence of high rates of infection/high antibody titres caused by/addressed towards a certain microorgansim (Mahoney and Ross, 1972).

Therefore, a certain infection occurring in conditions of endemic stability is characterised by low morbidity and low mortality in the population of the vertebrate host (Norval *et al*., 1992).

According to the existing literature on TBIs, in order to establish in field conditions, endemic stability would require: i) high prevalence of infection in the vector tick; ii) the presence of a durable vertebrate reservoir representing a steady source of infection for competent ticks; iii) high resistance to the emergence of

clinical disease in the vertebrate host, especially at a young age (Uilenberg, 1995; Deem *et al.*, 1996a; Jonsson *et al.*, 2012).

Therefore, for endemic stability to establish within a certain area, competent tick vectors of TBD-causing pathgens must be present in sufficient numbers to ensure regular infection ('challenge') of young animals by 9 months of age (Mahoney and Ross, 1972). This applies at least for anaplasmosis, babesiosis and cowdriosis, TBIs for which an age-dependent severity of disease (so-called 'inverse age resistance' or 'inverse age immunity') was demonstrated (Mahoney and Ross, 1972; Uilenberg, 1995; Jonsson *et al.*, 2008; Norval *et al.*, 1984). The concept of inverse age resistance consists indeed in the higher resitance or tolerance to an infection in young cattle (until approximately nine months of age) compared to adults in an endemic area (Mahoney and Ross, 1972; Uilenberg, 1995; Jonsson *et al.*, 2008; Norval *et al.*, 1984). This is possible as young stock are protected by passive colostral immunity in the first two months of their life, and later on (i.e. three to nine months of age) by innate immunity (Mahoney and Ross, 1972; Jonsson *et al.*, 2012).

Calves challenged in this period of the life usually develop only sub-clinical conditions (Mahoney and Ross, 1972; Jonsson *et al.*, 2012). Weaned cattle (older than at least six to nine months) will then be exposed to active infections, from which they can only recover following development of actively acquired immunity (Jonsson *et al.*, 2012).

The most important epidemiological features contributing to the establishment and maintenance of endemic stability for the three aforementioned TBIs are summarized herebelow.

Anaplasmosis – The following factors are pivotal for the existence of endemic stability with regards to anaplasmosis:

a) The presence of chronically infected (i.e. 'carrier') cattle, recovered from the acute infection, representing a reservoir for feeding boophilid ticks (Eriks *et al.*, 1989). As with cowdriosis, this is of great importance, considering that transovarial transmission of microorganisms does not occur in this case, as opposed to babesias (Bock *et al.*, 2004; Chauvin *et al.*, 2009);

b) Another key element is represented by the possibility for *A. marginale* and *A. centrale* to be transmitted mechanically by arthropods other than ticks (e.g. biting and sucking flies, etc.) and surgical fomites and needles (Aubry and Geale, 2011). This latter element, for instance, can considerably contribute to the spread of the infection within a confined area;

c) Moreover, *Anaplasma* species were shown to be able to experimentally infect foetuses *in utero* (Swift and Paumer, 1976; Zaugg and Kuttler, 1984; Zaugg, 1985). Young indigenous cattle are however less likely than adults to develop the disease, due to passive immunity gained from the colostrum, first and innate immunity after (Norval *et al.*, 1984; Egbe-Nwiyi *et al*., 1997; Jonsson *et al.*, 2012).

Babesiosis – Endemic stability is known to occur also for babesiosis (reviewed in Bock *et al.*, 2004), with the following factors playing a decisive role:

a) Animals recovered from the acute infection acting as carriers for a number of years (Mahoney, 1969; Johnston *et al.*, 1978; de Vos and Potgieter, 1994; Zintl *et al.*, 2005);
b) Transovarial infection in the vector ticks (i.e. *Boophilus* spp.), according to which a single infected engorged female can significantly contribute to the spread of the infection in a given area via her progenies (Bock *et al*., 2004). In this regards, tick infection rates, seemingly higher for *B. bigemina* than for *B. bovis* (Mahoney and Mirre, 1971), may be crucial for the establishment of endemic stability in a given area (Jonsson *et al.*, 2012);
c) Passively acquired immunity from the colostrum in calves (i.e. inverse age immunity) (Mahoney and Ross, 1972; Mahoney, 1974).

In Nigeria, in the 1920s it was suggested that cattle in endemic areas became infected in early life without displaying apparent infection and that acquire immunity was life-long due to repeated re-infections by ticks (Leeflang and Ilemobade, 1977a).

Cowdriosis – Endemic stability is known to occur for heartwater in most of SSA (Deem *et al*., 1996a**;** Bezuidenhout, 2009). The establishment of this epidemiological condition for this TBI relies essentially on:

a) Presence of chronically infected reservoirs, the carrier animals – Their epidemiological role compensates for the lack of transovarial transmission in infected *A. variegatum* (Deem *et al*., 1996a).

b) Vertical transmission from dams to calves – Intra-uterine and possibly also colostral vertical transmission of *E. ruminantium* infection (Deem *et al*., 1996b). Newborn infected calves can therefore readily serve as the source of infections for competent ticks (Deem *et al*., 1996a),

c) Resistance of calves to the clinical condition. This has been well documented to be possible due to several factors, such as: i) calves' reactive immunosystem, stimulated by the peri-natal infection (Banks, 1982; Osburn *et al*., 1982), ii) the presence of protective antibodies in the maternal colostrum until four weeks of age (Du Plessis, 1984; Deem *et al*., 1996b); iii) the appearance of innate immunity as soon as the colostral passive immunity vanishes (Deem *et al*., 1996a);

d) The routes of infections in the tick vectors – *E. ruminantium* transmission from ticks enables asymptomatic calves to become carriers of the infection and also ensures continuous exposure of older cattle to *E. ruminantium*, contributing to the maintenance of herd immunity to the infection (Deem *et al*., 1996a). Importantly, possible routes of transmission of the infections within *A. variegatum*, include not only the transstadial transmission from larvae to nymphs and from nymphs to adults, but also the intrastadial transmission occurring between adult ticks feeding on the same anatomical site of the same host forming typical clusters, due to the release of the AAAP (Bezuidenhout, 1987), but also via transfer of infected male ticks from one host to another susceptible naïve one (Andrew and Norval, 1989; Norval *et al*., 1990; Kocan *et al*., 1993). These aspects, together with its three-host biology, maximize the possibility for a single *A. variegatum* tick to transmit *E. ruminantium* infection.

Several authors have also postulated the existence to some extent of genetic resistance of certain indigenous cattle breeds, including the White Fulani, towards this disease (Uilbenberg, 1997), although at present there is a lack of experimental

evidence in this respect (Deem *et al*., 1996a) including for cattle of White Fulani breed.

1.2.4 Tick control

The term 'tick control' usually refers to the treatment of animals or the environment with devices that are able to reduce the challenge posed by certain target ticks (Walker, 2011). Accordingly, in this thesis, the definition of 'tick control' will refer to the treatment of livestock and specifically cattle, aimed at disrupting tick attachment, thus causing ticks to fall off their host (i.e. 'tick expellency'), and preventing further attachment of ticks to the host (i.e. 'tick repellency'), for a duration of time depedent on the 'residual activity' of the product (Halos *et al*., 2012). Therefore, tick control as herein described refers to halting of tick infestation and reduction in tick burden, with possible consequences on TBIs transmission.

The most common chemotherapeutic options currently available for the treatment of the TBIs aforementioned (i.e. anaplasmosis, babesiosis and cowdriosis), illustrated in the previous Sections 1.2.2.1.1–1.2.2.1.4), do not fall within the definition of 'control' utilized throughout this PhD thesis.

1.2.4.1 Chemical control in SSA – from arsenicals to pyrethroids

Control measures against livestock ticks and TBIs were initially devised, at the industrial level, towards the middle of the nineteenth century, due to the expansion of the livestock industry in tropical and sub-tropical countries in Africa, Latin America and Australia (reviewed in Graf *et al*., 2004). In these contexts, the idea of tick 'eradication', was meant as "the complete disappearance of target ticks from a given geographical area" (Walker, 2011), a concept which became progressively less conceivable and feasible, unless applied to circumscribed spaces like islands (reviewed in Walker, 2011).

At present, tick eradication seems also unattainable in most of SSA where the vast majority of cattle are still kept under pastoral management, and herd transhumance is a frequent practice (Latif, 1992). Therefore, over the past century, in

SSA as well as in the rest of the world, acaricides have played a major role in the control of ticks and TBIs (Young *et al*., 1988; Latif, 1992; Kocan, 1995).

The first chemical acaricide employed in Africa was arsenious oxide (AO), extensively used from the late 1800 (i.e. first report dated 1893 from Southern Africa) to the 1960s-70s and mainly administered in dipping vats (Norval *et al*., 1992; George *et al*., 2004). In South Africa, the use of AO-based dipping vats was implicated in the successful eradication of East Coast fever (ECF), caused by *Theileria parva* (Graham and Hourrigan, 1977; Lawrence, 1992).

Nonetheless, issues such as resistance in one-host ticks (i.e. *Rh*. (*Bo*.) *annulatus* and *Rh*. (*Bo*.) *decoloratus*) which was reported as emerging in 1935, the narrow safety limits for cattle, as well as concerns about presence of toxic residues in animal tissues, caused the withdrawal of synthetic organic insecticides in the decade after World War II (Graham and Hourrigan, 1977).

Organochlorines became available as a valid replacement only in the mid 1940s (Shaw, 1970). Dichlorodiphenyltrichloroethane (DDT) and benzenehexachloride (BHC) were the first compounds of this group of organic insecticides to be commercialized as acaricides (Cobbet, 1947; Maunder 1949; Whitnall *et al*., 1951), and were soon followed by other active ingredients (i.e. dieldrin, aldrin, cyclodiene, toxaphene), also widely employed for tick control in cattle (George *et al*., 2004).

However, organochlorines were also affected by the emergence of resistance, with several molecules of this class losing effectiveness against populations of *Rh.* (*Bo.*) *decoloratus*, *Rh.* (*Bo*.) *microplus* and *Rhipicephalus appendiculatus* across much of equatorial and Southern Africa (Whitehead, 1958; Baker and Shaw, 1965; Shaw, 1970). This, together with the issue of persistence of organochlorines in the environment as well in the food chain via the fat tissue (Ware, 2000), caused the withdrawal of these molecules from the market (Kunz and Kemp, 1994).

It was then that organophosphates (OPs) were developed. Amongst others, ethion, chlorpyrifos, chrlofenvinphos and coumaphos were the most widely used OPs against ticks (George *et al*., 2004). Currently considered as the least tolerable pesticides to vertebrates use nowadays (Ware, 2000), their toxicity had to be taken into account considering the possible oral ingestion by cattle when plunged in dip

vats or tanks (Walker, 2011). Nevertheless, OPs were more degraded in the environment and metabolized in the vertebrate tissues, allowing shorter slaughter-witholding periods compared to their predecessors (Graf *et al*., 2004).

Carbamate acaricides (e.g. carbaryl and promacyl) were their successors, displaying advantageous lower systemic and local (i.e. dermal) toxicity compared to the pesticides previously employed (Graf *et al*., 2004). However, their effectiveness was soon compromised by the emergence of cross-resistance with OPs (Roulston *et al*., 1968; Schntner *et al*., 1972; McDougall and Machin, 1988). On the whole, resistance issues for both OPs and carbamates have caused a significant reduction in their use in Africa, as well as in Latin America and Australia (Kunz and Kemp, 1994).

Formamidines (i.e. amitraz, clenpyrin, chlordimeform and chloromethiuron) were developed in the 1960s enabling farmers to overcome the resistance issues posed by the employment of OPs and carbamates (George *et al*., 2004). Besides dips (Stanford *et al*., 1981), amitraz in Africa has also been employed in spray (Morrow *et al*., 1993) and spot-on formulations (Peter *et al*., 2006).

Resistance to formamidines was first recorded in the late 1970s, though by then another class of molecules, the pyrethroids, had already been developed (Graf *et al*., 2004).

The first generation of synthetic pyrethrins or pyrethroids (i.e. allethrin, bioallethrin, tetamethrin, phenothrin, resmethrin, bioresmethrin and kadethrin) was developed in fact in 1949 via modification of the chrysanthemic acid of the natural pyrethrum, a compound extracted from the flower heads of crysanthemum (i.e. *Chrysanthemum cinerariifolium* and *Dalmatian pyrethrum*) (Anadón *et al*., 2009). Only the third generation of this class, though, included molecules (e.g. permethrin and fenvalerate) able to control cattle ticks (Davey and Ahrens, 1984; Ware, 2000). The last generation of pyrethroids was introduced at the end of the 1970s and included molecules (e.g. cypermethrin, cyfluthrin, deltamethrin, flumethrin, etc.) appreciated for being volatile thus able to display repellency also towards insects flying around treated animals (reviewed in Beugnet and Franc, 2012). Moreover, these compounds are known also for showing ‘residual (or persistent) activity’ (i.e. efficacy lasting for several days, up to weeks, after application, depending on

concentration and formulation of the active ingredient) when applied on the animal skin as well as on the environment (Beugnet and Franc, 2012).

Pyrethroids' spectrum of activity varied according to the molecules, with some (e.g. delatemthrin and permethrin) acting as both insecticides and acaricides, while others (e.g. flumethrin) mainly as acaricides (Stendel, 1985). For instance, flumethrin displays satisfactory toxicitiy against one- and multiple-host ticks at relatively lower concentrations than the other pyrethroids (e.g. deltamethrin and cis-permethrin) (Schnitzerling *et al*., 1989).

In SSA, so far pyrethroids have been used in several formulations including dips (Okello-Onen *et al*., 1994), foothbaths (Stachurski, 2006; Stachurski and Lancelot, 2006; Bouyer *et al*., 2007), spray (Torr *et al*., 2007), pour-on (Gouteux *et al*., 1996; Kok *et al*., 1996; Kamuanga *et al*., 2001; Rowlands *et al*., 2001; Okiria *et al*., 2002; Habtewold *et al*., 2004; Bekele *et al*., 2010) spot-on (Gouteux *et al*., 1996) and impregnated ear-tags (Young *et al*., 1985; Rechav, 1987; Schroder and van Schalkwyk, 1989).

1.2.4.2 The issue of resistance and the need for an 'integrated approach'

As described in the previous section, phenomena of acaricide resistance started emerging rather soon after the beginning of their widescale use (Graf *et al*., 2004). While the main drive of arthropocidal product development was initially represented by the search of spectrum of activity, later the pursuit of alternative modes of action that enabled the avoidaince of resistance became of priority (Graf *et al*., 2004). This was the case of organichlorines, commercialized to overcome resistance to arsenic compounds; OPs to substitute organochlorines; amidines to overcome the inefficacy of OPs; and pyrethroids to replace amidines (reviewed in Graf *et al*., 2004).

Pyrethroid resistance started being reported in the mid-1980s, spreading rapidly in the 1990s and allowing the re-introduction of amidines on the market (Graf *et al*., 2004). Soon, though, the re-use of amidines resulted in the re-apperance of resistance (Graf *et al*., 2004). However, this does not mean that all compounds within these classes are now ineffective (Graf *et al*., 2004).

In SSA (Luguru *et al.*, 1984; Regassa and de Castro, 1993; Mekonnen *et al.*, 2002; Lovis *et al.*, 2013), as well as in Australia (Nolan, 1980; Jonsson and Hope, 2007; Lovis *et al.*, 2013) and Latin America (Rosario-Cruz *et al*., 2009; Guerrero *et al.*, 2012; Lovis *et al.*, 2013) one-host boophilid ticks were the first to develop resistance due to their life cycle largely spent in close contact with the host thus with any acaricidal product applied onto its hair and skin (Davey and Ahrens, 1984; Walker, 2011; Lovis *et al.*, 2013). Importantly, regardless of the compound employed, it was soon clear that frequency of application had an implication with the emergence of resistance (Jonnson *et al*., 2000).

The issue of acaricide resistance was of such global reach, that the 'Veterinary Parasite Resistance Group' (VPRG) was formed in 1995 by members of the eight major animal health pharmaceutical companies (i.e. Merial, Pfizer, Schering Plough, Elanco, Novartis, Bayer, Intervet and Fort Dodge) to address this problem. The VPRG acted as an expert consultative group with the task of advising industrial and non-industrial organizations including the Food and Agriculture Organization (FAO) of the United Nations, on the implications and consequences, monitoring and management, of parasite resistance (Graf *et al*., 2004).

As an outcome of the increasing awareness, the adoption of 'integrated control' strategies was advocated in tropical and sub-tropical areas, to counteract acaricide resistance with economically and environmentally sustainable operations (Nolan, 1981; 1990). Often referred to as 'Integrated Pest Management' (IPM), integrated control consists of the systematic application of two or more methods to control ticks and the infections they transmit in a given livestock species (Bram, 1994; de Castro, 1997). The ultimate goal is to rely on a set of affordable measures in a way that, would one of them become ineffective, the control plan on whole would still make a positive impact (Graf *et al*., 2004).

With regards to Africa, the FAO has been recommending the adoption of the development of integrated control programmes and several countries (i.e. Burundi, Kenya and Zimbabwe) have seemingly started taking the first steps towards the implementation of such programmes (Norval *et al*., 1992).

Several elements could be potentially combined with each other as part of an integrated strategy (Young *et al.*, 1988; Graf *et al.*, 2004).

So far, the following have been most employed in field situations in SSA:

1.2.4.2.1 Rotation of pesticides

Already employed for several years in crop science (Zehnder *et al*., 2007) 'rotation' is a term applied to a control strategy that alternates the use over time of two or more chemicals with differing modes of actions and, ideally, without cross-resistance (Roush, 1993). The rotation scheme is based on the assumption that the frequency of resistant ticks within a population would decline during the time when the alternate chemical is used (Roush, 1993).

1.2.4.2.2 'Strategic' and 'threshold' control

According to the 'strategic' control, animals are treated with acaricides only in certain times of the year, when ticks are known to be most abundant (Pegram *et al*., 1995). The 'threshold' control is based on the use of acaricides only when the tick load on animals exceeds a "pre-defined, economically damaging number of adult ticks[3]" (Pegram *et al*., 1995).

1.2.4.2.3 'Restricted application' of acaricides

This approach was developed on the basis of data obtained from behavioural studies on ticks. It consists in limiting the application of the acarcides only to certain body areas, where the most hazardous ticks feed (see also Table 1.1).

For instance, knowledge that adults of certain species (e.g. *A. variegatum* and *Hyalomma* spp.) in SSA attach to the feet of livestock before moving to more permanent feeding sites (e.g. abdomen, groin, udder, scrotum, etc.) led to the development of acaricide-based foot-baths as alternative to the previous full dip tanks, successfully employed in Burkina Faso, not only for halting infestation by *A. variegatum* (Stachurski, 2006; Stachurski and Lancelot, 2006) but also for the control of tstetse-borne trypanosomiasis (Bouyer *et al*., 2009). Analogously, in Ghana, spray formulations of amitraz were applied fortnightly only to the *A.*

[3] Original quote from Pegram *et al*. (1995).

variegatum's predilection sites in cattle for the prevention of bovine dermatophilosis (Morrow *et al*., 1993).

Importantly, applying acaricides onto the hooves and the legs of cattle may also have an impact on immature stages of several genera usually parasitizing the lower quarters of the animals (see Table 1.1)

1.2.4.2.4 Exploitation of host resistance

It has long been known that indigenous Sanga and Zebu (*B. indicus*) cattle breeds and their crosses are innately less susceptible to tick infestation than taurine (*B. taurus*) breeds and their crosses (de Castro, 1997*;* Latif, 1993; Black *et al*., 2001; Naessens, 2006). Their resistance lies in the ability to respond immunologically to tick infestation, which is strongly heritable (Seifert, 1971; Oberem, 1984). This immunity is expressed by direct effects on tick feeding and survival on host, and it is such that the higher the tick burden the animal bears, the stronger the immunological response will be (Randolph, 1994). As a matter of fact, ticks feeding on resistant hosts have lower engorgement weights and egg production (Wikel and Whelen, 1986). Therefore, resistant indigenous *B. indicus* cattle tend to carry lighter tick burdens, with fewer completely engorged females and fully fed males than exotic *B. taurus* breeds (de Castro and Newson, 1993).

Moreover, this inheritable resistance is also coupled with physical and morphological (i.e. skin thickness, coat type and muscular consitution) as well as behavioural characteristics enabling a better contention of tick infestation. Short-haired and thick-skinned African breeds of cattle are very sensitive to the tactile stimula generated by ticks crawling up their body, evoking energetic flickering movement of their skin, made possible by their well developed subcutaneous muscular mass (Norval *et al.*, 1992).

Other behavioural responses include stamping of the hooves and other licking and grubbing patterns typical of grooming (Norval *et al*., 1992). Interestingly, indicus cattle seem also better capable to avoid clusters of *Rh.* (*Bo.*) *microplus* larvae questing on grass stems (Sutherst *et al*., 1986).

1.2.4.2.5 Pasture rotation (or spelling)

This strategy aims to reduce the host-finding rate in ticks by modulating the livestock density on pastures. Basically animals are moved from an area to another over a period of time to deny unfed ticks the opportunity to parasitize a host (Hernández *et al*., 2000; George *et al*., 2004).

Research has shown that field vacation should last for at least two years for eradicating from a target area *A. variegatum*, 15 months for *Rhipicephalus appendiculatus*, and 12–18 months for *Boophilus* spp. (reviewed in Walker, 2011). Therefore, while it may be feasibile for certain nomadic ethnic groups traditionally practicing the transhumance (e.g. the Fulani, see Sections 1.2.5, 1.2.6), this measure is however unpractical for smallholder farmers owning only limited plots of land (Walker, 2011). Even for mobile communities, though, it should be taken into account that pasture not suitable for tick development often lacks lush vegetation and nourishing grass (Walker, 2011) and that increasing population pressure further complicates the pursuit of favourable pastures (FAO, 2001).

Moreover, the planning of movement restriction of cattle or other livestock species as a practice of tick control, should take into account the possible role of hosts, especially for immature stages of two- to three- host target ticks, played by several wildlife species (Norval and Perry, 1990). The presence of suitable wildlife in an area kept cattle or livestock free may indeed thwart the accomplishment a control plan, regardless of the length of a vacation of a certain pasture (Norval and Perry, 1990).

1.2.4.2.6 Burning of the vegetation

Reduction of vegetation cover through farming and burning may provide a less favourable environment for tick development (de Castro, 1997), based on the assumption that desiccation is one of the major threats to tick development (especially for nymphs and adults) (Sonenshine, 1991).

1.2.4.2.7 Biological means of control ('Tick predators')

Smallholder African farmers usually exploit domestic chickens (*Gallus gallus domesticus*) for their capacity to feed on immature and adult ticks, from the environment as well as on cattle (Hassan *et al*., 1991).

Oxpeckers (*Buphagus* spp.) are more efficient and less opportunistic tick feeders than chickens, though these birds are undoubtedly less tameable than the former. Moreover, their value to farmers is still questionable, considering that, by feeding on the skin, they may exacerbate wounds created by ticks on cattle (van Someren, 1951; Bezuidenhout and Stutterheim, 1980). Nevertheless, lately the presence of oxpeckers in farmed areas of SSA has been facilitated by the use of acaricides less toxic to them, like those based on formamidines (Bezuidenhout and Stutterheim, 1980; Stutterheim and Brooke, 1981).

1.2.4.2.8 Immunization methods

Another example of an alternative method of control is represented by immunization of cattle against either ticks or tick-borne pathogens (reviewed in Merino *et al*., 2013).

1.2.4.2.8.1 Tick vaccines

Vaccination against ticks certainly is an attractive environmentally-friendly alternative for controlling tick infestation and preventing pathogen transmission. Targeting a common tick vector can theoretically enable to reduce, indirectly, the prevalence/incidence of several TBIs in a given area (reviewed in Merino *et al*., 2013).

The principle of anti-tick vaccination consists of targeting certain molecules (i.e. the 'antigens') of critical importance to tick survival, whose inhibition or disruption leads to irreversible alterations and even death of the infesting tick (reviewed in Willadsen, 2004). For this to happen, ticks need necessarily to attach to their host so that they can come into contact with their blood and immunoglobulins. Therefore, the main challenge inherent to the development of anti-tick vaccines lies in the identification of truly protective antigens in ticks. The refined and

continuously developing molecular tools currently available can certainly assist in this task (Merino *et al*., 2013). In particular, two groups of antigens could be targeted when conceiving an anti-tick vaccine: i) 'exposed' antigens, proteins contained in the tick's salivary secretions and that interact with the vertebrate host's immune system during the tick feeding process, and ii) 'concealed' antigens, which are not part of normal host-parasite interaction and do not evoke an immunological response under normal conditions (Nuttal *et al*., 2006).

Development of vaccines against the 'southern cattle tick' *Rh*. (*Bo*.) *microplus* commenced in the early 1980s and the first vaccines became commercially available in 1994 (reviewed in de la Fuente *et al*., 2007). In particular, two anti-tick vaccines are currently available to control the infestation by this tick species, namely Tick-GARD (Hoechst Animal Health, Australia; Queensland Dairy Farmers Organization, Australia) and Gavac (Heber Biotec S.A., Havana, Cuba) commercialized in Australia and Latin America (e.g. Cuba, Mexico, Brazil and Colombia), respectively (de la Fuente *et al*., 2007). Both vaccines are prepared using as antigen a recombinant protein (i.e. Bm86), consisting of 650 amino acids, usually bound to the surface of the digest cells constituting the gut membrane in the southern cattle tick (Gough and Kemp, 1993). The Bm86 is therefore an example of a 'concealed' antigen; cattle immunized against it produce antibodies that are ingested by feeding ticks, with consequent damage to the tick's gut due to the interaction of the immunoglobulins with gut membrane's proteins (Willadsen, 2004). Vaccination using the Bm86 antigen produces several advantageous effects including i) reduction in numbers of ticks feeding to maturity, ii) reduction in weight and reproductive capacity of female ticks and iii) increase of post-feeding mortality of adult ticks (de la Fuente *et al*., 1998; Willadsen, 2004).

As a result, the prevalence of some TBIs can also be affected. In particular, tick vaccines could affect the transmission of TBIs either i) by reducing the numbers of feeding ticks, thus influencing the overall transmission rate of a certain tick-borne parasite or ii) by altering the transmission efficiency of each individual tick due to the disruption of its physiological functions caused by the interaction of immunoglobulins with the gut membrane proteins (Willadsen, 2004).

Field data have shown a very significant reduction of the incidence of bovine babesiosis by *B. bovis* in Australia and Cuba, and *B. bigemina* in Australia (de la Fuente *et al*., 1998; Pipano *et al*., 2003). Interestingly, considering the role of mechanical vectors played by biting insects or contaminated fomites in the epidemiology of bovine anaplasmosis (Foil, 1989; Scoles *et al*., 2005; Aubry and Geale, 2011; Baldacchino *et al*., 2013), the Bm86 antigen-based vaccination has so far been able to control the transmission of *A. marginale* only in certain regions (e.g. parts of Cuba, Brazil and Colombia) where ticks are the main epidemiological vector of this rickettsia (de la Fuente *et al*., 1998).

Interestingly, in spite of its immunological properties, the biological function of the Bm86 protein has not been fully understood (Willadsen, 2004). From the earliest experiments it was clear that the Bm86-based vaccine had very little activity against larvae and only some effect on nymphs of *Rh.* (*Bo.*) *microplus* (Willadsen, 2004). The higher activity on adult stages seems to be related to their greater ingestion of blood from the host (Willadsen, 2004). Nonetheless, larvae hatching from eggs of females that fed on vaccinated cattle are seemingly less viable than normally (Willadsen, 2004). Consequently, considering the one-host biology of boophilid ticks and the effect of the vaccine on the tick's reproductive capacity, the greatest benefits of vaccination would usually become tangible from the second and even subsequent tick generation(s) (Willadsen, 2004). In Australia, therefore, the vaccine was commercialized with the recommendation to be combined with the strategic use of acaricides, and that the first vaccination would be given at the time of the year when tick numbers are the lowest (e.g. winter in temperate parts of Australia) (Willadsen, 2004). As a result, at least in the first few years after the adoption of the vaccine strategy, a way of measuring the efficiency of the vaccine became the evaluation of the reduction in acaricide usage (Willadsen, 2004). For example, in on-farm trials carried out in beef cattle properties in Australia, each booster vaccination enabled to save an average of 2.4 acaricide applications (Willadsen, 2004). Similarly, in Cuba, six years of heavily government-controlled vaccine use in over 500,000 cattle enabled to reduce acaricide treatments from an average of 15–17 times to 2.8 times per year and to increase the inter-treatment interval from 1–2 weeks to 12–25 weeks (de la Fuente *et al*., 1998). As a

consequence, the national acaricide consumption dropped from 480 to 80 tonnes per year, with over $6 million of savings in the cattle industry (de la Fuente *et al*., 1998). Even more interestingly, it has also been shown that macrocyclic lactones applied onto vaccinated cattle showed an improved efficacy of at least a ten-fold, possibly attributable to the increased penetration of the pesticide in the ticks due to the damage caused to tick's gut by the vaccine (Kemp *et al*., 1999).

Besides being environmentally-friendly, another great advantage of the use of anti-tick vaccines is the unlikelihood of being subjected to development of resistance by ticks (Willadsen, 2004). In fact, the increase of selection of acaricide-resistant ticks is causing an increase of vaccine demand in certain regions, such as Mexico (de la Fuente *et al*., 2007).

Nonetheless, the protectiveness of the Bm86-based vaccine is affected by strain variation of the ticks' Bm86 amino acid sequence, causing variability in protection of cattle populations depending on geographic areas (Willadsen, 2004). For example, in South America, the Bm86-based vaccine showed efficacies ranging between 51% and 88% using Cuban and Mexican isolates of *Rh*. (*Bo*.) *microplus* respectively, while it showed almost no efficacy when using a tick isolate from Argentina (i.e. the so-called 'A strain') (de la Fuente *et al*., 1999; García-García *et al*., 1999). For this reason, the Argentinian Bm86, differing for 21 amino acid substitutions in the sequence of the mature Bm86 protein, was later on renamed 'Bm95' (García-García *et al*., 2000).

Furthermore, the Bm86 vaccine is mainly effective against boophilid ticks (Merino *et al*., 2013). Besides *Rh*. (*Bo*.) *microplus*, both Tick-GARD and Gavac have shown an efficacy of almost 100% against *Rh*. (*Bo*.) *annulatus* (Fragoso *et al*., 1998; Pipano *et al*., 2003). Interestingly, in contrast to *Rh*. (*Bo*.) *microplus*, the Bm86-based vaccine shows a considerable activity against both larval and nymphal stages of *Rh*. (*Bo*.) *annulatus*, with positive repercussions on the prevention of the transmission of *B. bovis*, mainly vectored by these stages of boophilids (Mahoney and Mirre, 1979).

However, very low to zero efficacy was recorded in trials employing this vaccine against the African ticks *A. variegatum* and *Rh. appendiculatus* (de Vos *et al*., 2001).

Another negative aspect of employing only concealed antigens for the development of anti-tick vaccines is the fact that natural tick infestation is unlikely to stimulate the immune response, considering that the host's immune system is usually not exposed to these parasite's antigens. This would therefore require a frequent boosting through vaccination (Willadsen, 2004).

The aforementioned drawbacks highlight the need for improving the composition and formulation of the currently existent anti-tick vaccines, possibly including several antigens, of both exposed and concealed type.

Although the proteins characterized so far may already include suitable vaccine candidates, at present there is paucity of reports available on their assessment in vaccination trials (Willadsen, 2004). Thus far, tick vaccines have never been commercialized in SSA, though research efforts addressed towards the identification of tick-protective antigens have been put by a network of several institutions from Africa (i.e. École Nationale de Médecine Vétérinaire, Tunisia, and Ministry of Food and Agriculture, Ghana; University of Pretoria, South Africa), Europe (i.e. Utrecht University, The Netherlands, and University of Castilla la Mancha, Spain), and Australia (i.e. Commonwealth Scientific and Industrial Research Organisation, CSIRO)[4] (Nijhof, 2010).

1.2.4.2.8.2 Vaccines against tick-borne pathogens

In SSA, the most renowned example in this respect is represented by the 'infection-and-treatment method' (ITM), employed for the immunzitation of cattle against East Coast fever (ECF) by *Theileria parva* (Di Giulio *et al*., 2009). Based on the inoculation of a certain load of live infective stages (i.e. sporozoites) of the parasite, to be followed by the administration of long-acting oxytetracycline, so far the ITM has been successfully employed in some parts of East Africa (e.g. Tanzania and Uganda) (reviewed in Di Giulio *et al*., 2009). The most commonly used

[4] Wellcome Trust project 075799 entitled 'Adapting recombinant anti-tick vaccines to livestock in Africa' under the 'Animal Health in the Developing World' initiative.

formulation of the ECF 'vaccine' consists of the so-called 'Muguga cocktail', a trivalent combination of *T. parva* isolates originating from three different localities in Eeast Africa, namely Kiambu, Muguga and Serengeti (Radley *et al*., 1975). The protection conferred by this vaccine is largely affected by the variation of *T. parva* strains circulating in ECF-affected areas (Di Giulio *et al*., 2009).

In West Africa, where ECF does not occur due to the absence of its principal vectors (i.e. *Rhipicephalus appendiculatus* and *Rhipicephalus zambesiesis*) (Norval *et al*., 1999), ITM attempts were carried out for the prevention of heartwater, but they were never applied to large-scale field conditions due to the existence of different *E. ruminantium* strains hampering the effectiveness of this approach (Sumption, 1996).

1.2.5 The Fulani pastoralist system

The livestock population bred in the Plateau mainly consists of cattle, followed by goats, sheep, chickens, ducks, turkeys, and a relatively small number of swine (Lee, 1972). Historically, the total number of cattle reared in the whole of Plateau State exceeds one million head (Bourn *et al*., 1992). They are mainly owned by the Fulani pastoralists (Awogbade, 1979).

Nationwide considered as 'the custodians' of bovine herds, the Fulanis engage in cattle rearing as their primary activity, secondary to which is agriculture (Iro, 1994). Livestock rearing represents their major source (i.e. 60%) of income, followed by crops (i.e. 25%) and milk production (i.e. 15%) (Majekodunmi, 2011) (Figure 1.14). The Fulani usually own the land they settle in, which is primarily used for agriculture purposes, besides the construction of their homesteads (Majekodunmi, 2011). Fulani's livestock are mostly grazed on common/unused land, being tethered within their owner's parcels only at night (Majekodunmi, 2011).

Figure 1.14 – Fulani herdman milking his cow in the morning (Village of Mangar, Bokkos LGA, Plateau State).

Especially in the wet season, the Fulani tend to capitalize on the abundance of rain and dung to plant corn, millet, sorghum, and home gardens in their backyards. The main purpose of farming is to overcome the shortfalls during the periods of drought, and possibly provide livestock with crop residues.

When surveyed in 2011, the size of the cattle herds within the Fulani communities varied from seven to 5,015 head, with an average of 250 cattle per household (Majekodunmi, 2011). Female cattle are usually more numerous than bulls or oxen, constituting, on average, the largest part (>70%) of each herd population (Majekodunmi, 2011). The higher proportion of females reflects the importance of dairy farming compared to the scant use of bulls for plowing or draft power (Pullan, 1980).

Cattle are therefore mostly bred to supply milk and other dairy products such as yoghurt (Awogbade, 1979). In fact, except for the case of old repeatedly calved females or incurably sick or sterile individuals, meat production comes preferably from the slaughtering of small ruminants (e.g. West African Dwarf goats and sheep) rather than from cattle (Cisse, 1980). Importantly, indeed, meat does not represent

the mainstay of the Fulani diet, which is mainly based on the consumption of vegetables (i.e. cassava, corn, millet, sorghum, sugarcane), rice, wheat, nuts and fruits (Iro, 1994).

All Fulani cattle in the Plateau are of the autochthonous (i.e. *B. indicus*) genotype, with a vast majority (approximately 80%) belonging to the White Fulani breed, which has long been appreciated for its high milk yields (White and Wickens, 1976; see also Figure 1.14), and a small number of Bunaji brown cattle and White Fulani x Bunaji brown crosses, which are primarily used to improve the meat yields (Dongkum[5], personal communication) (Figure 1.15).

Figure 1.15 – White Fulani and (brown) Bunaji cattle from the same herd (Village of Bokkos, Bokkos LGA, Plateau State).

Factors such as the poor adaptability of exotic (i.e. *Bos taurus*) species to the environment, their high susceptibility to diseases, demand for large grazing areas and

[5] Dongkum, Charles; 'Stamp Out *Sammore* (SOS)' programme, fieldwork manager, principal livestock technologist at the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos, Plateau State, Nigeria.

rarely available supplementary feeding, have historically discouraged the Fulani from undertaking species diversification (Awogbade, 1979).

Most cattle in the Plateau are grazed under an extensive pastoral system, largely affected by the fluctuation of seasons conditioning the accessibility to water provisions and suitable pastures (Awogbade, 1979). Although all of the Fulani on the Plateau own their homesteads, paucity of fodder and water sources may cause their temporary displacement during the dry season, moving their herds southward to the district of Gwantu in the neighbouring state of Kaduna, which provides ideal pastures during the critical months of the year (i.e. from end of September to May, latest) (Pullan, 1980). The Fulani herdsmen usually let their cattle graze on Gwantu's crop residues until the beginning of the following wet season. When the weather conditions change and tsetse and other harmful fly swarms start expanding in the more southern districts, the Fulani will then lead their herds back to the Plateau (Awogbade, 1979). Inevitably, the more mobile a Fulani community is, the less likely they will be dedicated to agriculture (Iro, 1994).

1.2.6 Tick control on the Nigerian Plateau

In general, the use of short interval (e.g. every one week) application of acaricides, widespread in Eastern and Southern Africa, has never been largely adapted in West Africa (Sumption, 1996). Though dipping tanks and spraying races had been established in Nigeria since the babesiosis outbreak in Katsina, in the northern part of the country, in the 1960s, the Fulani herders were reported to be repeatedly reluctant to adopt chemical control, regarded as too burdensome and time consuming, depriving their cattle of precious grazing opportunities (Leeflang and Ilemobade, 1977a). Neither dip tanks nor acaricides have been therefore successfully established in Plateau State (Pullan, 1980; Maina, 1986).

Usually known as '*kaska*' in the local Fulbe language, ticks are perceived by the Fulani on the Plateau as a hazard to their cattle (Otufale and Adekoya, 2012). These pastoralists have historically preferred traditional methods of control, such as the manual removal of ticks (so-called 'de-ticking') (Bayer and Maina, 1984),

carried out three times a week during the wet season (i.e. April to October) and twice a week during the dry months (i.e. November to March) (Pullan, 1980; Maina, 1986).

This method, however, is far from effective, as usually only adult ticks are removed, especially the larger sized *Amblyomma* and *Hyalomma* spp., while most immature stages and smaller sized *Rhipicephalus* and *Boophilus* spp. still remain attached (Maina, 1986). In particular, the Fulani recognize the most conspicuous *Amblyomma* ticks as 'koti' ('hazardous'), whereas other smaller species (e.g. boophilid ticks) as 'miri' ('less harmful') (Bayer and Maina, 1984). Furthermore, as the collection is usually performed while the animals are standing, the least easily accessible body parts (e.g. ear canals, axillae, groin, tail base, etc) get less accurately cleared of ticks (Bayer and Maina, 1984). In addition, although it keeps animals free from 'tick worry', hand removal of ticks may not help prevent the transmission of TBIs when not performed on a daily basis, as the transmission of pathogens may occur within 24–36 hours after the attachment of these arthropods to their hosts (Piesman and Spielman, 1980; Bezuidenhout, 1987; Azad and Beard, 1998).

Besides adopting the 'de-ticking' practice, the Fulani cattle keepers try to minimize the risk of tick infestation in their cattle by reducing the time spent at watering points where they suspect vectors (especially tsetse flies) are most likely to occur. Moreover, in the wet season, they delay the grazing of their herds until late in the morning, as they consider tick infestation on the grass to be the highest in the early hours (Maina, 1986).

Fires are also burnt next to the cattle pens to keep away ticks and biting insects at night (Maina, 1986).

1.3 Thesis aims

Therefore, aims of this PhD research were the following:

1. To document and update the existing knowledge on the composition of tick species infesting cattle in Central Nigeria, assessing the average infestation rate of animals in the study area, also according to age classes (i.e. calves, juveniles and adults);
2. To implement a molecular method enabling the sensitive simultaneous detection of all possible tick-borne microorganisms infecting cattle in West Africa;
3. To then assess the occurrence of TBIs of both veterinary and zoonotic importance in cattle from Central Nigeria employing the aforementioned molecular method;
4. To assess the longitudinal effect of a pyrethroid-based control programme, part of the SOS initiative, on the kinetics of TBIs in indigenous cattle in Central Nigeria;
5. Based on points 1, 3 and 4, provide suggestions for the devising of possible sustainable and realistic strategies for controlling tick infestation in cattle in Central Nigeria, for the improvement of livestock health and productivity.

The present thesis was therefore structured according to the following chapters, developed to fulfil the aforementioned aims:

- Chapter 1: provides global information on the study area and a review on ticks and TBIs of cattle in Nigeria, as well as on the control methods historically employed in Africa and particularly in Nigeria.
- Chapter 2: intends to fulfil aim no. 1.
- Chapter 3: intends to fulfil aim no. 2 and 3.
- Chapter 4: intends to fulfil aim no. 4.
- Chapter 5: reviews results obtained in Chapters 2–4 and fulfils aim no. 5, drawing conclusions on the applicability of the knowledge generated in this thesis to field conditions in the study area.

- Appendix / Section 6: provides an extended version of Table 3.8 (i.e. Table 6.1), together with a review of the history of the development of the reverse line blotting (RLB) method and of its application to the diagnosis of TBIs in SSA, with an overview of the adavantageous aspects related to its use. Two published articles pertaining to the field of research presented in this thesis and a poster advertising the RLB summer course held at Utrecht University in summer 2013, are also included in this section.

Chapter 2 – Ixodid ticks of traditionally managed cattle in Central Nigeria

2.1 Introduction

Knowledge on tick distribution is an essential requisite for devising any effective control of these arthropods and the diseases they transmit (de Castro, 1997). At present, however, the existing information available on the tick fauna infesting cattle in Nigeria is rather outdated (Dipeolu, 1975a; Bayer and Maina, 1984; Iwuala and Okpala, 1978; Moammed, 1977), and mostly generated from studies carried out in the south of the country (Dipeolu, 1975a; Iwuala and Okpala, 1978). With special reference to Central Nigeria, the only work published to date predominantly focused on the seasonal dynamics of *Amblyomma variegatum*, without identifying any further than the genus level the other specimens retrieved (Bayer and Maina, 1984). Moreover, as indicated in Chapter 1, West African cattle are currently threatened by the expansion of the harmful and invasive tick species, *Rhipicephalus* (*Boophilus*) *microplus*, seemingly imported from Brazil, and retrieved to date in the Ivory Coast (Madder *et al*., 2007), Benin (Madder *et al.*, 2011, 2012; De Clercq *et al*., 2012), Burkina Faso, Mali and Togo (Adakal *et al*., 2013). Ascertaining the occurrence of *Rh. (Bo.*) *microplus* in this area is of importance, as this species is known for being the most competent vector of the hazardous bovine pathogen *Babesia bovis* (Bock *et al*., 2004), and also for displaying acaricide resistance (Baffi *et al*., 2008).

2.2 Aims

The aim of this study was therefore to document the composition of tick species infesting cattle in Central Nigeria, assessing the infestation rate of surveyed animals, at a time of the year (i.e. wet season) when the tick load on the host is known to be most abundant (Bayer and Maina, 1984).

Generating such data would be essential to orientate further diagnostic endeavours aiming to detect cattle TBDs as well as for the design of area-specific control strategies, especially needed in the event of introduction of exotic taurine breeds.

2.3 Materials and Methods

2.3.1 Plateau State

Often simply indicated as 'the Plateau', The Plateau State (9°00´–10°30´ N; 8°30–09°30´ E), occupies an area of approximately 26,899km^2 in the central part of Nigeria (Lee, 1972; www.plateaustate.gov.ng) (Figure 2.1).

This part of Nigeria is often referred as the 'middle belt', a human geographical term indicating a region of Central Nigeria characterised by a great heterogeneity of ethno-linguistic groups, encompassing several states such as Adamawa, Benue, Kogi, Kwara, Nasarawa, Niger, Plateau and Taraba, together with the southern parts of the more northern States of Bauchi, Borno, Gombe, Kaduna and Kebbi (Adekunle, 2004). Culturally, this is an area of dynamic cultural transition between the mostly Islamic north and the mostly Christian south of Nigeria (Adekunle, 2004).

Figure 2.1 – The Plateau State (highlighted in green), located within Nigeria's middle belt (source: www.downandrowmore.org).

With a population of about 3.5 million inhabitants hosted within 17 local government authorities (LGAs), the Plateau shares boundaries with four other federal states, namely Bauchi, Kaduna, Nassarawa and Taraba. The Plateau's capital, Jos, is the only city of the state, founded in the early twentieth Century (i.e. 1906) by miners following the discovery of lodes of tin (www.plateaustate.gov.ng).

Due to its geographical location, the Plateau State is characterised by an extreme ethnic diversity including the 53 groups of autochthonous origins, amongst which the largest are the Birom, Ngas and Taroh, and those that historically migrated here coming from the South (e.g. Igbo, Yoruba, etc.) and the North of country (e.g. Hausa, Fulani, etc.) as a result of mining and/or trading and livestock rearing (www.plateaustate.gov.ng).

The economy of the Plateau is mainly based on mining, agriculture and livestock farming, with the latter two activities being of major importance to the rural areas (Lee, 1972).

2.3.2 Study area

The study was carried out in the second half of September 2010 in 9 villages belonging to three neighbouring LGAs, namely Bokkos, Mangu, and Pankshin, in the central part of the Plateau State, Nigeria (Figure 2.2). On the whole, the three LGAs within which the 9 study villages were included covered an area of 42 km^2, ranging between 9°14’ and 9°59’ N of latitude and 8°79’ and 9°38’ E of longitude, at an average altitude of 1,280 m.

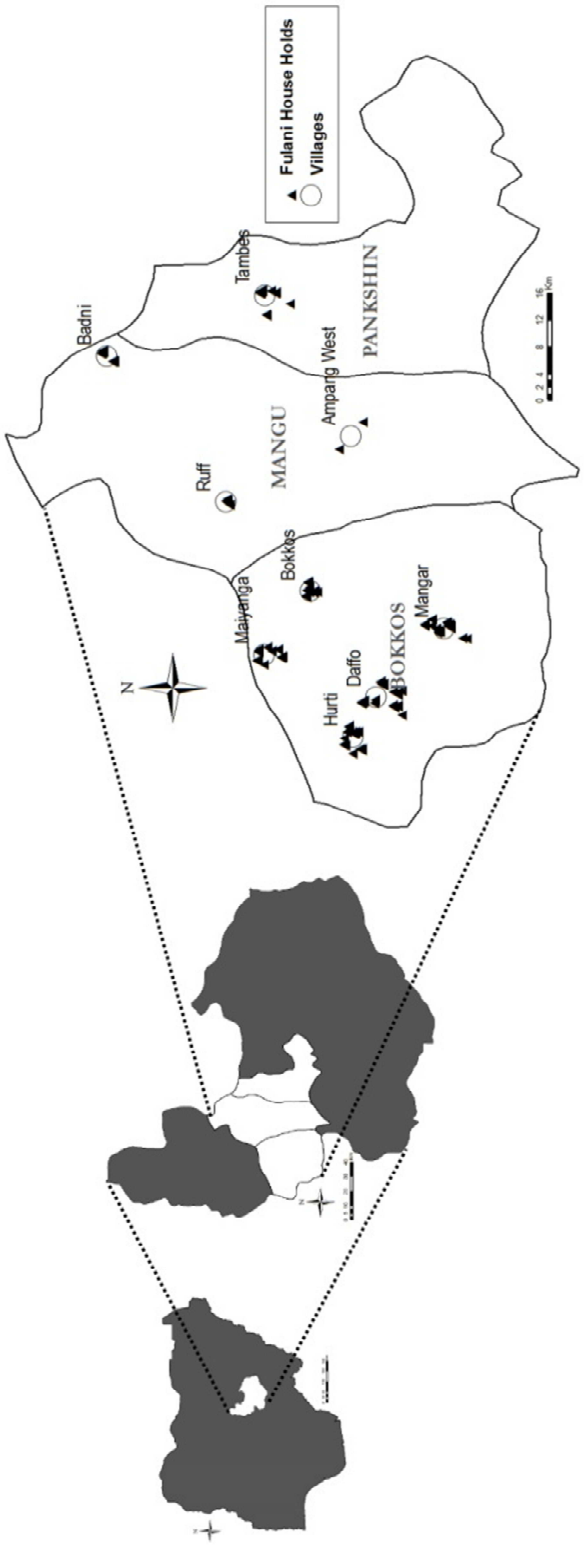

Figure 2.2 – Map of the study area.
(a) = Federal republic of Nigeria; **(b)** = Plateau State;
(c) = local government areas with the nine villages where the sampling took place.

All study villages are included in the sub-humid region of Nigeria, with the dry season generally extending from November to April, and the wet season from April-May to October (Figure 2.3). The rainfall pattern is mono-modal, with most (~80%) of the rains occurring between June and September. Annual rainfall is of ~1,400 mm and the daily mean temperature ranges between 18 and 22°C (Odumodu, 1983).

Figure 2.3 – Typical landscape of the Plateau during the wet season.

Vegetation is typical of the Northern Guinea Savanna woodland, with wide grasslands punctuated by rocky hills and occasionally by afforested areas. Several grass species are found in the bushland and scrub forest including *Carissa edulis*, *Dalbergia hostilis*, *Diospyros abyssinica*, *Diospyros ferrea*, *Dodonaea viscosa*, *Euphorbia desmondii*, *Euphorbia kamerunica*, *Euphorbia poissonii*, *Ficus glumosa*, *Kleinia cliffordiana*, *Rhus longipes*, *Rhus natalensis*, *Ochna schweinfurthiana*, *Olea capensis*, *Opilia celtidifolia* and *Pachystela brevipes* (White, 1983). Various trees are maintained around the rural settlements including canarium (i.e. *Canarium schweinfurthii*), *Cassia* spp., oil palm (i.e. *Elaeis guineensis*), *Euphorbia kamerunica*, *Euphorbia poissonii*, *Eucalyptus* spp., mango (i.e. *Mangifera indica*). The most common tree species in the remaining woodland are *Berlinea* spp., *Isoberlinia doka*, *Lannea schimperi*, *Syzygium guineense*, *Uapaca somon* and *Vitex doniana* (Lee, 1972). Cassava (i.e. *Manihot esculenta*), cocoyam (i.e. *Colocasia* spp.), guinea corn (i.e. *Sorghum bicolor*), maize (i.e. *Zea mais*), millets (i.e. *Panicum*

spp.) and potatoes (*Solanum tuberosum*) are the main crops cultivated in the area (Lee, 1972).

All cattle reared in the area are of autochthonous (i.e. *B. indicus*) genotype, with a vast majority (~80%) belonging to the White Fulani breed, and a small number to either Bunaji or to White Fulani x Bunaji crossbreeds. Cattle graze under natural communal pasture year-round according to the traditional Fulani herding system (Figure 2.4).

Figure 2.4 – Indigenous White Fulani cattle extensively grazed on the Plateau in the lush vegetation of the wet season (village of Bokkos, September 2010).

Other livestock species raised in the area include sheep, goats, poultry, and dogs. Wildlife includes several rodent species such as giant rats (i.e. *Thryonomys swinderianus*), hedgehogs (i.e. *Atelerix* spp.), squirrels (i.e. *Anomalurus derbianus*, *Anomalurus beecrofti*, *Xerus erythropus*) and hares (i.e. *Lepus capensis*). Baboons (i.e. *Papio* spp.) may be seen in the rocky hills and red patas (i.e. *Erythrocebus patas*) inhabit the areas of thick vegetation. Bird species comprise white-throated francolins (i.e. *Peliperdix albogularis*), guinea fowls (i.e. *Agelastes* spp.), and the very common cattle egrets (i.e. *Bubulcus ibis*) (Lee, 1972).

No chemical acaricidal control is applied under the Fulani farming system, either to any livestock species or to the environment.

2.3.2 Tick sampling and identification

During the sampling days, all cattle reared in each village were brought to a gathering site previously identified at the village level. Gathering sites were chosen in agreement with the local Fulani community, mainly based on the sites' accessibility to all cattle keepers and their herds. As an incentive for cattle keepers, at the same time when tick collection took place, all cattle were treated according to a worming protocol consisting of an oral administration of boluses of a broad spectrum anti-helminthic containing a combination of 60% Oxyclozanide and 40% Levamisole Hydroclhoride (Kepxan Bolus, Kepro®, Holland).

Sampling usually started at 7 am.

In each village, systematic random sampling was carried out, starting from the herds gathered the closest to the homesteads' conglomerates, and ending with those gathered the furthest. Cattle were selected at equal intervals, sampling one every three animals (i.e. first animal sampled, second and third not sampled, fourth cattle sampled). The first animal to be sampled was chosen randomly.

In each village, all visible adult ticks were collected from at least 15 randomly selected cattle varying in age and sex, all belonging to indigenous (i.e. *B. indicus*) White Fulani breed. Tick collection was performed using blunt steel forceps, by thorough examination of the entire body surface of the animals (Figure 2.5); ticks from each animal were stored separately in vials containing 70% ethanol, labelled with information on the host (i.e. sample number and age of the animal), village, and date of sampling.

Figure 2.5 – Tick collection being performed (village of Maiyanga, Bokkos LGA).

Age of the animals was estimated on the basis of the dentition score method developed for zebu cattle under a low plane of nutrition (Kikule, 1953) and on the information provided by their owners. Once quantified, each animal's age was recorded either as 'calf' (0-6 months), 'juvenile' (6-24 months), or 'adult' (older than 24 months). Once in the laboratory, all collected ticks were counted and identified to the genus and species level using a Vickers® stereomicroscope reaching up to 100x magnification and following the morphological keys of Walker *et al*. (2003). For those belonging to the genus *Rhipicephalus*, keys by Walker *et al*. (2000) were also used.

2.3.3 Statistical analysis

Prevalence of each tick species was calculated with the exact binomial 95% confidence interval (CI) using the reciprocal of the sample size through the 'survey' package in the R software (http://www.R-project.org). Mean tick counts and their standard error were calculated for each village, age group and tick species.

For each tick species, total counts recorded at each village level were 'weighed' according to the reciprocal of the sample size (knowing the total cattle population of each village).

Cumulative counts were statistically compared according to age groups of cattle by the use of the Kruskal-Wallis test. Post hoc analysis was then performed using the Holm P value adjustment method in the pairwise Wilcoxon rank sum test.

Study villages were compared statistically on the basis of the mean tick burdens of infestation using a one-way analysis of variance (ANOVA) test with Geisser-Greenhouse correction, with the software Prism 6.05 for Windows (GraphPad Software, Inc.). When the one-way ANOVA test detected a significant difference ($p<0.05$) amongst the groups (i.e. villages) examined, multiple comparisons were computed with the same software using the Turkey method, to enable the comparison of the mean tick burden of each village with that of every other village. This approach was used taking into account the mean tick infestation obtained from each study village considering the three age classes (i.e. calves, juveniles and adults) altogether as well as the mean tick infestations recorded considering age classes separately (i.e. adults or juveniles or calves).

Moreover, for each study village, a two-tailed t-test with Welch's correction was performed using the software Prism 6.05 for Windows (GraphPad Software, Inc.) to assess the occurrence of any significant difference between the mean infestation obtained considering the three age classes (i.e. calves, juveniles and adults) altogether and that of each age class considered alone.

For all aforementioned statistical tests, p values lower than 0.05 were considered as statistically significant.

2.4 Results

A total of 228 cattle were checked for tick infestation in 9 villages (average=25 animals/village). On average, each tick collection, performed examining scrupulously the overall body surface of the animals, lasted for 10-15 minutes.

On the whole, a good compliance of cattle keepers was recorded in most villages. Besides the worming treatment, all Fulani herdsmen welcomed the removal of ticks from their cattle, though carried out for surveying purposes. However, due to the longer time required for tick collection compared to the worming protocol, in all villages, some of the herds gathered towards the outmost parts of the sampling sites were, once dewormed, brought away by their owners. This occurred mainly due to the increasing atmospheric temperature as the sun kept rising, hence the need for the herdsmen to release their cattle to graze in the pastures. In some other instances, some cattle were brought away from the sampling sites due to the necessity of their herders to attend religious functions. Nevertheless, in all villages the systematic sampling procedure was carried out on all available animals.

To evercome the discrepancy between the total cattle population and the actual number of cattle sampled, the reciprocal of the sample size of each village was considered when estimating the prevalence of each tick species, taking into account the ratio between their total cattle population and the number of animals surveyed per village (as explained in Section 2.3.3).

2.4.1 Tick burdens

The population sampled consisted of 14 calves, 33 juveniles, and 181 adults. All animals were infested with adult ticks. A total of 5,011 adult ixodid ticks (i.e. 1,935 males, 3,076 females) were collected (Table 2.1). Mean tick counts recorded per village were relatively high (i.e. 22 ± 1.4), ranging from 7.6 (± 1.5) in Ampang West to 46.5 (± 7.91) in Mangar (Table 2.1).

Table 2.1 – Number of cattle sampled, cumulative tick counts, mean tick loads ± standard error (SE) according to the villages of sampling.

Village Name	No. of Cattle Sampled	Total Cattle Population	No. of Ticks Collected	Mean Tick Count/animal ± SE
Ruff	15	154	322	21.5 ± 3.65
Mangar	15	1,373	697	46.5 ± 7.91
Tambes	16	854	301	18.8 ± 4.4
Daffo	21	2,933	686	32.7 ± 5.6
Ampang West	22	790	168	7.6 ± 1.5
Hurti	23	1,011	594	25.8 ± 4.3
Badni	27	383	851	31.5 ± 3.3
Bokkos	36	2,142	608	16.9 ± 2.1
Maiyanga	53	2,543	784	14.8 ± 2.4
Total	**228**	**12,183**	**5,011**	**22 ± 1.4**

A rather high individual variability was seen in terms of tick load, depending on the age and size of the animals.

Calves were found to be significantly less infested than adults (p=0.004), whereas no statistically significant difference was found comparing adults with juveniles (p=0.2). Although not statistically significant (p=0.2), the average proportion of ticks infesting juveniles was higher than the mean loads on calves (Table 2.2).

Table 2.2 – Cumulative (total) tick counts and mean tick loads ± standard error (SE) of cattle according to age groups.

Age Group	Cumulative Counts	Mean Tick Load ± SE
Calves (< 6 months)	142	10.1 ± 2.7[a]
Juveniles (6-24 months)	595	18.0 ± 2.9
Adults (> 24 months)	4,274	23.6 ± 1.6[a]

[a] Statistical significance between the two age groups.

2.4.2 Tick burdens according to study villages

Results obtained from the one-way ANOVA test carried out comparing the mean tick burdens of the study villages considering the three age classes (i.e. calves, juveniles and adults) altogether, are summarized in Table 2.3, highlighting the existence of a significant difference ($p < 0.0001$) among the villages from the study area.

Table 2.3 – Results of the one-way ANOVA test carried out taking into account the overall mean tick burden of each study village, obtained considering animals of all age classes altogether.

One-way ANOVA test considering all animals	
P value	< 0.0001
F value	8.367
R squared value	0.2341

In particular, the following villages were found to differ significantly for from each other:

- Ampang West and Badni ($p=0.0003$);
- Ampang West and Daffo ($p=0.0004$);
- Ampang West and Hurti ($p=0.03$);
- Ampang West and Mangar ($p<0.0001$);
- Maiyanga and Badni ($p=0.005$);
- Maiyanga and Daffo ($p=0.006$);
- Maiyanga and Mangar ($p<0.0001$);
- Mangar and Bokkos ($p<0.0001$);
- Mangar and Hurti ($p=0.02$);
- Mangar and Ruff ($p=0.007$);
- Mangar and Tambes ($p=0.001$).

Results of the one-way ANOVA test carried out considering only adult cattle from each village are summarized in Table 2.4, highlighting, also in this case, the existence of a significant difference ($p<0.0001$) between the mean tick burdens recorded in the villages from the study area.

Table 2.4 – Results of the one-way ANOVA test carried out taking into account the mean tick burden of adult cattle of each study village.

One-way ANOVA test considering only adult cattle	
P value	<0.0001
F value	8.366
R squared value	0.2801

In particular, the following villages were found to differ significantly from each other:

- Ampang West and Badni ($p=0.006$);
- Ampang West and Daffo ($p=0.0006$);
- Ampang West and Mangar ($p<0.0001$);
- Badni and Bokkos ($p=0.01$);
- Badni and Maiyanga ($p=0.01$);
- Bokkos and Daffo ($p=0.001$);
- Bokkos and Mangar ($p<0.0001$);
- Daffo and Maiyanga ($p=0.001$);
- Mangar and Hurti ($p=0.005$);
- Mangar and Maiyanga ($p<0.0001$);
- Mangar and Ruff ($p=0.003$);
- Mangar and Tambes ($p=0.0009$).

Table 2.5 provides, for each village, an overview of the mean tick counts recorded on the adult cattle population as well as that recorded considering all three age classes (i.e. calves, juveniles and adults) altogether. P values obtained from the two-tailed t-test are also provided.

Table 2.5 – Number of adult cattle sampled in each study village, adults' mean tick counts, overall mean tick counts and p values resulting from the two-tailed test carried out comparing the two means.

Village Name	**No. of adult cattle / total cattle sampled**	**Adults' Mean Tick count ± SE**	**Overall Mean Tick Count/animal ± SE**	**P value from two-tailed t-test**
Ruff	12 / 15	21.6 ± 3.8	21.5 ± 3.65	1
Mangar	10 / 15	53.8 ± 10.9	46.5 ± 7.91	0.6
Tambes	14 / 16	20.5 ± 4.9	18.8 ± 4.4	0.8
Daffo	15 / 21	40.33 ± 6.9	32.7 ± 5.6	0.4
Ampang West	14 / 22	9.8 ± 2.1	7.6 ± 1.5	0.4
Hurti	22 / 23	26.4 ± 4.5	25.8 ± 4.3	0.9
Badni	20 / 27	34.6 ± 3.8	31.5 ± 3.3	0.5
Bokkos	32 / 36	15.3 ± 1.7	16.9 ± 2.1	0.6
Maiyanga	42 / 53	16.3 ± 2.9	14.8 ± 2.4	0.7
Total	**181 / 228**	**23.6 ± 1.6**	**22 ± 1.4**	

Results of the one-way ANOVA test carried out considering only juvenile cattle from each village are summarized in Table 2.6, showing in this case no significant difference between the mean tick burdens recorded in the villages from the study area (p=0.09).

Table 2.6 – Results of the one-way ANOVA test carried out taking into account the mean tick burden of juvenile cattle of each study village.

One-way ANOVA test considering only juvenile cattle	
P value	0.09
F value	1.991
R squared value	0.3892

Table 2.7 provides, for each village, an overview of the mean tick counts recorded on the juvenile cattle population as well as that recorded considering all three age classes (i.e. calves, juveniles and adults) altogether. P values obtained from the two-tailed t-test are also provided.

Table 2.7 – Number of juvenile cattle sampled in each study village, juveniles' mean tick counts, overall mean tick counts and p values resulting from the two-tailed test carried out comparing the two means.

Village Name	No. of juvenile cattle/ total cattle sampled	Juveniles' Mean Tick count ± SE	Overall Mean Tick Count/animal ± SE	P value from two-tailed t-test
Ruff	2 / 15	28.5 ± 24.5	21.5 ± 3.65	0.8
Mangar	2 / 15	41.0 ± 13.0	46.5 ± 7.91	0.8
Tambes	2 / 16	7 ± 0.0	18.8 ± 4.4	0.02*
Daffo	6 / 21	13.5 ± 3.8	32.7 ± 5.6	0.001*
Ampang West	2 / 22	4.5 ± 1.5	7.6 ± 1.5	0.2
Hurti	1 / 23	13	25.8 ± 4.3	0.007*
Badni	6 / 27	25.0 ± 6.2	31.5 ± 3.3	0.4
Bokkos	4 / 36	29.5 ± 13.0	16.9 ± 2.1	0.4

Village Name	No. of juvenile cattle/ total cattle sampled	Juveniles' Mean Tick count ± SE	Overall Mean Tick Count/animal ± SE	P value from two-tailed t-test
Maiyanga	8 / 53	8.9 ± 2.7	14.8 ± 2.4	0.12
Total	**33 / 228**	**18.0 ± 2.9**	**22 ± 1.4**	

*Significant differences between mean tick infestations.

Results of the one-way ANOVA test carried out considering only the calf population sampled are summarized in Table 2.8, highlighting, as for the overall cattle population and the adults, the existence of a significant difference (p=0.003) between the mean tick burdens recorded in the villages from the study area.

Table 2.8 – Results of the one-way ANOVA test carried out taking into account the mean tick burden of calves of each study village.

One-way ANOVA test considering only calves	
P value	0.003
F value	8.2
R squared test	0.75

In particular, the differences between the following villages resulted as significant:

- Mangar and Ampang West (p=0.001);
- Mangar and Maiyanga (p=0.03);
- Mangar and Badni (p=0.03);
- Mangar and Ruff (p = 0.02).

Table 2.9 provides, for each village, an overview of the mean tick counts recorded on the calf population sampled as well as that recorded considering all three age classes (i.e. calves, juveniles and adults) altogether. P values obtained from the two-tailed t-test are also provided.

Table 2.9 – Number of calves sampled in each study village, calves' mean tick counts, overall mean tick counts and p values resulting from the two-tailed test carried out comparing the two means.

Village Name	No. of calves/ total cattle sampled	Calves' Mean Tick count ± SE	Overall Mean Tick Count/animal ± SE	P value from two-tailed t-test
Ruff	1 / 15	6	21.5 ± 3.65	0.001*
Mangar	3 / 15	25.7 ± 6.7	46.5 ± 7.91	0.08
Tambes	0 / 16	-	18.8 ± 4.4	-
Daffo	0 / 21	-	32.7 ± 5.6	-
Ampang West	6 / 22	3.7 ± 1.1	7.6 ± 1.5	0.04*
Hurti	0 / 23	-	25.8 ± 4.3	-
Badni	1 / 27	8	31.5 ± 3.3	<0.0001*
Bokkos	0 / 36	-	16.9 ± 2.1	-
Maiyanga	3 / 53	9.7 ± 2.3	14.8 ± 2.4	0.1
Total	**14 / 228**	**10.1 ± 2.7**	**22 ± 1.4**	

*Significant differences between mean tick infestations.

2.4.3 Tick genera and species

Three tick genera (i.e. *Amblyomma*, *Hyalomma*, *Rhipicephalus* including *Boophilus* sub-genus) and 11 species were identified. *Rhipicephalus* (*Boophilus*) *decoloratus* (Koch, 1844) was the most prevalent species (41.4%), followed by *Rhipicephalus* (*Boophilus*) *annulatus* (Say, 1821) (15.4%); *Rhipicephalus guilhoni* Morel & Vassiliades, 1963 (12%); *Rhipicephalus* (*Boophilus*) *geigyi* (Aeschliman & Morel, 1965) (8%); *Hyalomma truncatum* Koch, 1844 (7.4%); *Amblyomma variegatum* (Fabricius, 1794) (6%); *Rhipicephalus simus* Group Koch, 1844 (4%); *Rhipicephalus turanicus* Pomerantsev, 1936 (1%); *Rhipicephalus sanguineus* (Latreille, 1806) (0.3%) and *Hyalomma rufipes* Koch, 1844 (0.2%) (Table 2.10).

Only one male specimen of *Rhipicephalus lunulatus* Neumann, 1907 was retrieved. 4% of adult specimens were identified as *Rhipicephalus* (*Boophilus*) spp. due to damaged mouthparts not allowing their morphological recognition any further than the sub-genus level (Table 2.3, Figure 2.6).

No *Rh.* (*Bo.*) *microplus* (Canestrini, 1888) was found in the study area. All three boophilids, *H. truncatum*, and *A. variegatum* were retrieved in all 9 villages. Male specimens outnumbered females for most species except for the boophilids and *Rh. sanguineus* (Table 2.10).

Table 2.10 – Cumulative counts, prevalence, number of females and males, and male : female ratio of ticks identified.

Tick Species	Total	Mean Prevalence % (95% Confidence Interval)	Males	Females	Male : Female Ratio
Rh. (*Bo.*) *decoloratus*	1,890	41.4 (36.5–46.3)	473	1,417	1 : 3
Rh. (*Bo.*) *annulatus*	819	15.4 (11.9–19.0)	189	630	1 : 3.3
Rhipicephalus guilhoni	434	12.0 (7.2–16.8)	302	132	2.3 : 1
Rh. (*Bo.*) *geigyi*	306	7.6 (6.1–9.1)	45	261	1 : 5.8
Hyalomma truncatum	681	7.4 (5.8–9.0)	469	212	2.2 : 1
Amblyomma variegatum	361	6.3 (4.5–8.1)	245	116	2.1 : 1
Rhipicephalus (*Boophilus*) spp.	205	4.1 (2.9–5.4)	12	193	1 : 16
Rhipicephalus simus Group	239	4.0 (2.5–5.5)	155	84	1.8 : 1
Rhipicephalus turanicus	39	1.2 (0.5–2.0)	22	17	1.3 : 1
Rhipicephalus sanguineus	10	0.3 (0.03–1.0)	4	6	1 : 1.5
Hyalomma rufipes	26	0.2 (0.1–0.4)	18	8	2.2 : 1
Rhipicephalus lunulatus	1	<0.1 (0.0–0.0)	1	-	1 : 0

The broadest diversity of tick species was encountered on adults, followed by juveniles, and calves. Boophilids, *H. truncatum*, and *A. variegatum* were more abundant in adults compared to juveniles and, more markedly, to calves (Table 2.11).

Table 2.11 – Cumulative and mean counts ± standard error (SE) of tick species according to age groups of cattle.

Tick Species	Cumulative Counts			Mean Tick Load ± SE		
	Calves	Juveniles	Adults	Calves	Juveniles	Adults
Rh. (*Bo.*) *decoloratus*	56	197	1637	4.0 ± 1.2	6.0 ± 1.7	9.0 ± 0.9
Rh. (*Bo.*) *annulatus*	16	104	699	1.1 ± 0.5	3.1 ± 0.7	3.9 ± 0.4
Rhipicephalus guilhoni	27	54	353	1.9 ± 1.3	1.6 ± 0.7	1.9 ± 0.6
Rh. (*Bo.*) *geigyi*	7	27	72	0.5 ± 0.2	0.8 ± 0.2	1.5 ± 0.2
Hyalomma truncatum	15	87	79	1.1 ± 0.4	2.6 ± 0.8	3.2 ± 0.4
Amblyomma variegatum	12	42	307	0.9 ± 0.4	1.3 ± 0.2	1.7 ± 0.2
Rhipicephalus (*Boophilus*) spp.	1	32	72	0.1 ± 0.1	1.0 ± 0.4	0.9 ± 0.1
Rhipicephalus simus Group	7	33	99	0.5 ± 0.4	1.0 ± 0.5	1.1 ± 0.2
Rhipicephalus turanicus	1	10	28	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1
Rhipicephalus sanguineus	0	7	3	0	0.2 ± 0.2	0.02 ± 0.01
Hyalomma rufipes	0	2	24	0	0.1 ± 0.04	0.1 ± 0.04
Rhipicephalus lunulatus	0	0	1	0	0	0.01 ± 0.01

2.5 Discussion

The distribution of ticks within a certain habitat depends on several environmental and climatic factors such as annual rainfall, atmospheric temperature and relative humidity (RH), vegetation cover, altitude and host availability (Sonenshine, 1993). This study was carried out in the late wet season, when the RH in the environment as well as the vegetation luxuriance, and therefore the abundance of adult ticks on cattle, are expected to be at their peak in Central Nigeria (Bayer and Maina, 1984; Iwuala and Okpala, 1978; Maina, 1986).

This study aimed to assess the species diversity of ticks infesting cattle and their burdens, in an area of Central Nigeria where cattle are reared extensively in absence of any acaricide-based treatment, in spite of the high tick challenge known to occur in the wet season (Bayer and Maina, 1984).

Tick counts and identifications carried out in this study only focused on the adult stages of these arthropods. Because of their small size, a large number of immature ticks can be easily overlooked during field collection, resulting into a biased estimate of counts. In addition, larvae and nymphs of most genera lack the neatly distinctive morphological features considered for identification to the species level. Therefore, counts of adults can be considered as representative of the total infestation of all instars over the year, especially for three-host tick species, whose immature instars feed for short periods (e.g. four days) on cattle as well as on other hosts (e.g. small ruminants, wildlife and birds)

Tick collection, especially if carried out examining the total body surface of cattle, is a rather lengthy process, lasting for 10 to 30 minutes for each adult cattle examined in the surveyed area, depending on their degree of infestation. As the tick sampling started from the animals gathered in closest proximity to the homesteads' conglomerates, cattle kept at the periphery of the sampling sites had to wait the longest before being sampled. This led to some compliance issues, with some herds leaving the gathering sites after being dewormed, without being subjected to tick sampling. This inevitably affected the total number of animals surveyed in this study.

Nonetheless, as indicated in Sections 2.3.3 and 2.4, to evercome the discrepancy between the total cattle population of the study area and the actual

number of cattle sampled, the reciprocal of the sample size of each village was considered when calculating the prevalence of each tick species.

On the whole, an average of 25 randomly selected cattle at each of the 9 villages were selected (Table 2.1). The higher number of adult rather than younger animals sampled reflects the age composition of Fulani herds, with at least 60% of cattle being adults (Maina, 1986).

2.5.1 Overall tick burdens

The overall mean tick load recorded (i.e. 22 ± 1.4) was considered as relatively high in the light of the hand-picking (or 'de-ticking') practice described in Chapter 1 (see also Section 1.2.6), which has likely reduced the actual number of adult ticks on the cattle sampled (see Figure 2.6).

Figure 2.6 – Young Fulani herders from the Plateau removing ticks manually from their cattle.

It is also possible that the transhumance of weaned cattle according to the traditional Fulani herding might also play a role in containing tick burdens as grazing areas are naturally spelled.

On the whole, the overall mean tick count recorded in the study area (i.e. 22 ± 1.4) was very much influenced by the infestation burden of adult cattle (i.e. 23.6 ±

1.6), the great majority of the animals sampled in this study (n=181/228, 79%). In fact, in none of the study villages the difference between the mean infestation of adults and the overall mean infestation (obtained considering the three age classes altogether) was significant (see Table 2.5). This was not the case for juvenile cattle and for calves. In several villages, the mean tick burden recorded for animals of these two age classes was significantly lower (halved or even smaller, $p<0.05$) than the mean infestation rate obtained considering the three age classes altogether (see also Tables 2.7 and 2.9).

The contribution of tick counts recorded in adult cattle to the overall mean tick infestation recorded is further proven by the fact that 10/12 significant differences recorded comparing villages for tick burdens in adult cattle, were also detected considering the three age classes (i.e. calves, juveniles and adults) altogether. Moreover, p values, F values and R squared values recorded executing the one-way ANOVA test considering the overall mean infestation and the adult cattle's mean infestations were very similar (see Tables 2.3 and 2.4).

All the most hazardous tick species were still recorded, although with different abundances, in all age groups (see Table 2.11), in all study villages, with potentially great implications in terms of infection transmission.

In particular, this study revealed a pronounced effect of host age and size on the number of infesting adult ticks, especially when comparing calves (< 6 months) with adult cattle (>24 months of age) (Table 2.2). Although with no statistical significance, the mean tick loads of calves were also found at a lower proportion than those of juvenile cattle (6–24 months old), which bore lower burdens than the adults (Table 2.2).

This finding is of interest especially considering that a conspicuous amount of calving in the Fulani herds takes place the early wet season (i.e. April-May) (Maina, 1986) and therefore the calves sampled in this study have likely lived through the entire rainy season, in presence of a high tick challenge. The significantly lower tick loads observed in calves as opposed to adults corroborates similar work carried out on indigenous cattle in SSA (Jongejan *et al.*, 1987; Marufu *et al*., 2011) including Nigeria (Iwuala and Okpala, 1978).

The lower tick burdens recorded in calves could be due to a combination of factors, including some form of innate immunity of indigenous cattle that decreases with age (Wikel and Bergman, 1997), the persistent grooming of calves by their respective dams (Fivaz and de Waal, 1993), and the smaller body surface of younger animals compared to adults (Mooring *et al*., 2000). It could be argued, indeed, that animals with larger surface areas would allow more contact opportunities for the ticks to attach. This is also predicted by the body size principle, according to which the smaller the animal the fewer parasites (i.e. engorging ticks) it can afford to accumulate per unit of body surface because of the greater body surface to mass ratio (Mooring *et al*., 2000). Moreover, the lower tick burden recorded in young animals could also be due to the Fulani's practice of maintaining calves for significant time apart from the adult cattle, tethered together close to the homesteads (see Figures 2.7 and 2.8). They therefore spend limited time grazing in the open grasslands with their dams, and are consequently less exposed to the higher parasite burdens found on the pastures, propelled by the higher host density.

Figure 2.7 – Calves tethered in proximity of the homesteads before being released to be nursed by their dams.

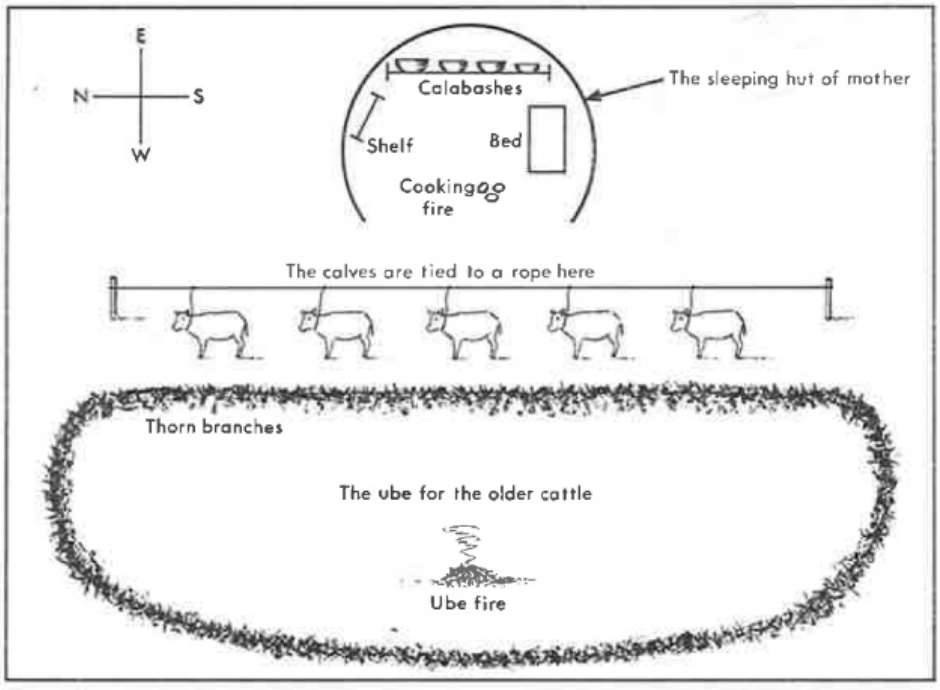

Figure 2.8 – Typical arrangement of a Fulani homestead. Note the calves tethered in the vicinity of the shelter (illustration by Oguntonade EO, from Tiffen, 1968).

2.5.2 Tick burdens according to study villages

Considering the mean tick burdens of the three age classes of cattle altogether, three villages presented significant differences with others, namely Ampang West, Maiyanga and Mangar.

The village of Ampang West was characterized by a very low mean tick burden (i.e. 7.6 ± 1.5) compared to the overall mean of 22 ± 1.4. In particular, the mean tick burden of Ampang West was significantly lower than that recorded in Mangar (i.e. 46.5 ± 7.91, $p<0.0001$), Badni (i.e. 31.5 ± 3.3, $p=0.0003$), Daffo (i.e. 32.7 ± 5.6, $p=0.0004$) and Hurti (i.e. 25.8 ± 4.3, $p=0.03$).

The remarkable difference of mean tick burdens recorded in Ampang West compared to other study villages can be attributed to several reasons. First of all, this could be due to the large number of calves sampled in this village (n=6 out of the total of 14 sampled in the whole study). In the case of Ampang West, calves represented nearly the 30% (i.e. 27%) of the total number of cattle sampled (n=22).

However, besides calves (3.7 ± 1.1), also other age classes of cattle from Ampang West presented considerably lower infestation rates than those documented in other villages, including both juvenile (4.5 ± 1.5) and adult cattle (9.8 ± 2.1).

The village of Ampang West is more isolated compared to other villages (see Figure 2.2) and hosts a medium to low cattle population (n=790, while the average in the study area was of 1,354 ± 324.8). These two elements may have contributed to the lower tick infestation of animals considering that tick presence on pastures depends on host densitiy (Sonenshine, 1993).

Overall mean tick burdens from the village of Maiyanga (i.e. 14.8 ± 2.4) resulted also significantly lower than those from other study villages, namely Mangar (i.e. 46.5 ± 7.91, $p<0.0001$), Badni (i.e. 31.5 ± 3.3, $p=0.005$) and Daffo (i.e. 32.7 ± 5.6, $p=0.006$). The lower mean burden recorded in Maiyanga was mainly affected by the mean infestation rate of its adult cattle (i.e. 16.3 ± 2.9), also significantly lower than that of adult cattle in the villages of Mangar (i.e. 53.8 ± 10.9, $p<0.0001$), Daffo (i.e. 40.33 ± 6.9, $p=0.001$) and Badni (i.e. 34.6 ± 3.8, $p=0.01$). Though significantly less infested than those from Mangar (i.e. 25.7 ± 6.7, $p=0.03$), calves from Maiyanga

presented with infestation rates (i.e. 9.7 ± 2.3) very close to the overall mean estimate of calves (i.e. 10.1 ± 2.7).

Located in the proximity of the northern border of Bokkos LGA, in the western part of the central Plateau, the village of Maiyanga hosts a population of 2,543 cattle. The largest number of animals sampled in this study was obtained in this village (n=53/228, 23%). Considering the high density of villages and of cattle in the surroundings, as well as the homogeneity of the agro-ecological conditions in the study area, it is supposed that the lower tick burden recorded in cattle from Maiyanga could be possibly attributed to management factors, including, among others, a more accurate removal of ticks by the Fulani pastoralists. This, however, may still not be reflected by a lower prevalence of bovine TBIs, considering that the tick species of greatest veterinary concern (i.e. *A. variegatum*, *H. truncatum* and the three boophilids) were found in all study villages, Maiyanga included.

Mangar was the village in which the highest mean tick count was recorded (i.e. 46.5 ± 7.91) being this higher than twice the overall mean tick count recorded in the study area (i.e. 22 ± 1.4). The mean tick count recorded in Mangar was significantly higher than that of Bokkos (i.e. 16.9 ± 2.1, $p<0.0001$), Tambes (i.e. 18.8 ± 4.4, $p=0.001$), Hurti (i.e. 25.8 ± 4.3, $p=0.02$), Ruff (i.e. 21.5 ± 3.65, $p=0.007$) and, as aforementioned, Maiyanga (i.e. 14.8 ± 2.4, $p<0.0001$) and Ampang West (i.e. 7.6 ± 1.5, $p<0.0001$). The only two villages compared to which the mean tick count of Mangar was not significantly higher were Daffo (mean tick count: 32.7 ± 5.6, $p=0.4$) and Badni (i.e. 31.5 ± 3.3, $p=0.2$).

With a total cattle population of 1,373 animals, the village of Mangar is located in the LGA of Bokkos, in rather close proximity to the village of Daffo. Importantly, the village of Mangar falls within the migration route of cattle coming from the north-eastern part of the Plateau State, moving in south-west-bound direction. This is therefore an area where the density of cattle on pastures is usually high and cattle may easily come in contact with herds from neighbouring villages as well as from other parts of the Plateau, favouring a high tick abundance on the pastures (Sonenshine, 1993) and possibly exchange of ticks between animals. Interestingly, in the village of Daffo, which is the closest to Mangar (see also Figure 2.2), a considerable overall mean tick count was also recorded (i.e. 32.7 ± 5.6), being this

still higher than the mean tick count recorded for the overall study area (i.e. 22 ± 1.4). This is considered as a reflection of the exposure of cattle to high tick challenge in this area of Bokkos LGA.

The mean infestation of adult cattle from the village of Mangar (i.e. 53.8 ± 10.9) was remarkable, being inevitably significantly higher than that recorded in adult cattle from Ampang West (i.e. 9.8 ± 2.1, $p<0.0001$), Bokkos (i.e. 15.3 ± 1.7, $p<0.0001$), Maiyanga (i.e. 16.3 ± 2.9, $p<0.0001$), Tambes (i.e. 20.5 ± 4.9; $p=0.001$), Ruff (i.e. 21.6 ± 3.8, $p=0.007$) and Hurti (i.e. 26.4 ± 4.5, $p=0.02$).

Importantly, the mean tick count recorded on calves in the village of Mangar (i.e. 25.7 ± 6.7) was higher than the mean recorded in the study area (i.e. 10.1 ± 2.7). Although the total number of calves sampled in this study was limited (n=14/228, 6%), the one-way ANOVA test showed a significant difference ($p=0.003$) among study villages in which calves were sampled (n=5/9). In particular, calves from Mangar were found significantly more infested than those from Ampang West (i.e. 3.7 ± 1.1, $p=0.001$), Maiyanga (i.e. 9.7 ± 2.3, $p=0.03$), Badni (i.e. 8, $p=0.03$), and Ruff (i.e. 6, $p=0.02$). This further highlights the high tick challenge to which cattle of all class ages are exposed in this village.

Though no significant difference was detected by the one-way ANOVA test when comparing juvenile cattle from the study area ($p=0.09$, see also Table 2.6), the mean infestation recorded in juvenile cattle from Mangar was still considerable (i.e. 41.0 ± 13.0) being very close to the mean infestation rate recorded for this village considering the three age classes altogether (i.e. 46.5 ± 7.91, $p=0.8$).

In the case of Mangar, ecological factors and vegetation conditions are considered less likely to play a role in determining this high tick burden, given the homogeneity of the agro-ecological zone in which these villages are located.

Adult cattle from the village of Bokkos (mean tick burden: 15.3 ± 1.7) were found significantly less infested than those from the villages of Daffo (i.e. 40.33 ± 6.9, $p=0.001$) and Badni (i.e. 34.6 ± 3.8, $p=0.01$), as well as Mangar (i.e. 53.8 ± 10.9, $p<0.0001$), as aforementioned. As stated for the village of Maiyanga, and considering the proximity of Bokkos to this village (see Figure 2.2), the differences recorded in the mean tick infestation of adult cattle are more likely attributable to management factors (e.g. a more thorough manual removal of ticks carried out by the

local herdsmen) instead of environmental or ecological conditions. Moreover, it should be considered that, except for that with Mangar, the differences of mean infestation of adult cattle from Bokkos with those from Daffo and Badni did not generate significant differences between these villages when considering the overall mean infestation of cattle of all ages. This is very likely due to the rather high mean tick burden documented in juvenile cattle from Bokkos (i.e. 29.5 ± 13.0), above the mean recorded for juveniles in the overall study area (i.e. 18.0 ± 2.9).

Possible genetic factors of resistance (e.g. intrinsic to the White Fulani breed) are not taken into account to justify the lower infestation rates recorded in villages differing significantly from others (i.e. Ampang West, Maiyanga and, to a lesser extent, Bokkos), considering the homogeneity of breed composition among the herds reared in the study area, with the majority (~80%) of cattle belonging to the White Fulani breed, and a smaller number being either Bunaji or White Fulani x Bunaji crossbreeds (see also Section 3.3.1).

2.5.3 Overview of tick genera and species

The study ascertained the presence of a rather broad variety of tick species infesting cattle in Central Nigeria, belonging to three genera (i.e. *Amblyomma*, *Hyalomma*, *Rhipicephalus* spp.) included in the family Ixodidae. Five out the 11 species identified (i.e. *Rh.* (*Bo.*) *decoloratus*, *Rh.* (*Bo.*) *annulatus*, *Rh.* (*Bo.*) *geigyi*, *H. truncatum* and *A. variegatum*) were retrieved in all study villages (data not shown).

2.5.3.1 Boophilids

Rh. (*Bo.*) *decoloratus* was the most abundant species in the area, in accordance with previous work (Dipeolu, 1975). The Nigerian Plateau seemingly provides an ideal environment for *Rh.* (*Bo.*) *decoloratus*, preferring highlands and sub-highlands receiving more than 800 mm of rainfall annually (Pegram *et al*., 1981). The second most prevalent species in this study was *Rh.* (*Bo.*) *annulatus*, previously found to be the most predominant tick parasitizing cattle in eastern Nigeria (Iwuala and Okpala, 1978). In Africa, the distribution of this tick is restricted

to the northern and western part of the continent (Walker *et al*., 2003). South of the Sahara, *Rh.* (*Bo.*) *annulatus* is associated with lowland rainforest and secondary grassland, with a clear increase in the vegetation cover after July-August (Estrada-Peña *et al*., 2006).

Both *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *annulatus*, transmit *B. bigemina* (Bock *et al*., 2004), *Anaplasma marginale* and *Anaplasma centrale* (Aubry and Geale, 2011), known to be endemic in Nigeria (Leeflang and Ilemobade, 1977a,b). Boophilids are one-host ticks that entirely develop on cattle after the egg hatch, their population is expected to be relatively constant throughout the year in this setting (Iwuala and Okpala, 1978), thus representing a constant threat with reference to the appearance of bovine anaplasmosis and babesiosis.

This study also ascertained the occurrence of *Rh.* (*Bo.*) *geigyi* in Central Nigeria. This species, merely of West African pertinence, was considered to be mainly distributed in the savanna and forest zones of Southern Nigeria, where it is the most abundant boophilid in the early dry season (Dipeolu, 1975). As this tick requires higher mean temperatures than *Rh.* (*B*o.) *decoloratus* and *Rh.* (*Bo.*) *annulatus* (Estrada-Peña *et al*., 2006), it is inferable that the cooler conditions of the Plateau and, more in general, of the northern Guinea savanna woodland, might hinder the expansion of its population in Central-Northern Nigeria. Although poorly investigated to date in terms of infection transmission, *Rh.* (*Bo.*) *geigyi* could be of veterinary relevance in Nigeria, where it was proven to harbour piriform 'vermicules' associated for shape and size with *B. bovis*, in both eggs and larvae that eventually infected splenectomised calves (Akinboade and Dipeolu, 1981).

A number (n=205) of boophilids were identified only as *Rhipicephalus* (*Boophilus*) spp. due to partial rupture of their mouthparts, likely to have occurred at the time of collection, considering the small size and the short rostrum of these ticks. In particular, these were mostly engorged female specimens (see Table 2.3), whose feeding state did not allow the objective assessment of morphological features (e.g. shape of genital aperture), other than the mouthparts. The rostrum of boophilids bears species-specific features, such as the teeth (denticle) rows in the hypostome and palp articles (Walker *et al*., 2003; see also Figure 2.9). In all cases, however, it was still possible to rule out the presence of *Rh.* (*Bo.*) *microplus* amongst these

specimens either for the number of denticle rows detected (i.e. three rather than four per column) or for the morphology and structure of the palps with regards to the following features:

- shape and extension of the internal margin of article I: much longer and concave in *Rh.* (*Bo.*) *annulatus* than in *Rh.* (*Bo.*) *microplus*;

- protuberance with or without an intact pectinate seta (present in *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *geigyi* and absent in *Rh.* (*Bo.*) *annulatus* than in *Rh.* (*Bo.*) *microplus*;

- shape of palp's article three: elongated and triangular-like in *Rh.* (*Bo.*) *annulatus*; truncated and trapezoid-like in *Rh.* (*Bo.*) *microplus*.

The damage in their hypostome, though, did not allow the discrimination between *Rh.* (*Bo.*) *decoloratus* (i.e. three rows of denticles) and *Rh.* (*Bo.*) *geigyi* (i.e. four rows of denticles, like *Rh.* (*Bo.*) *annulatus*, see Figure 2.9, a-b), thus limiting the definitive identification to the sub-genus level.

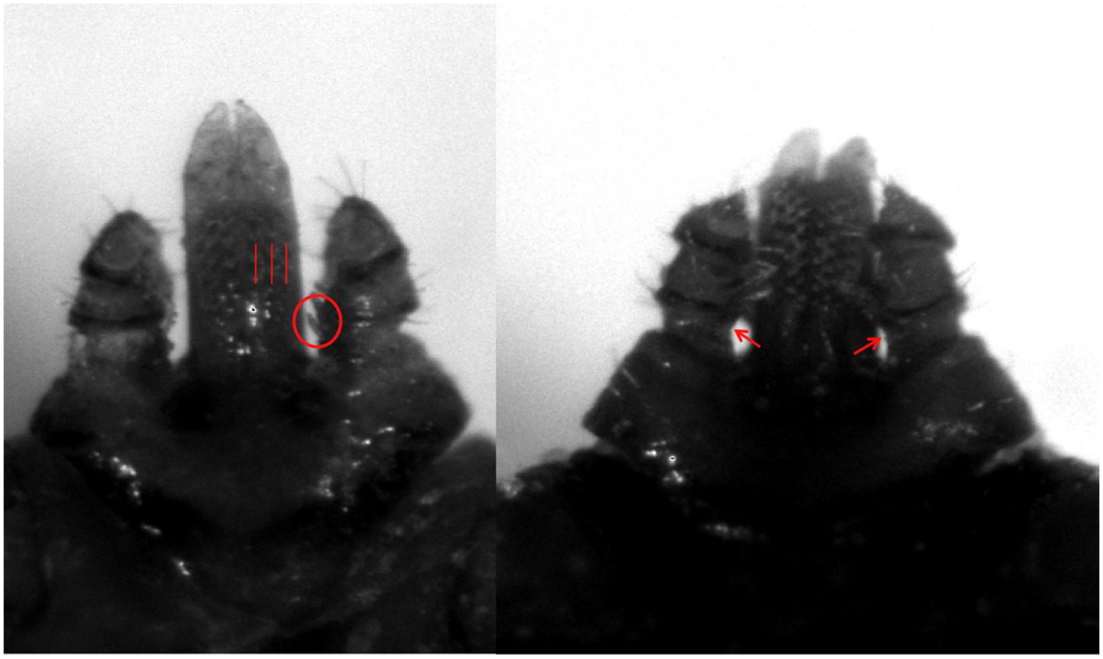

Figure 2.9 – *Basis capituli* of *Rh.* (*Bo.*) *decoloratus* **(a)** and *Rh.* (*Bo.*) *annulatus* **(b)**. Note the presence of three rows of denticles (red lines) in the hypostome and a protruding seta on the article I of the palp of *Rh.* (*Bo.*) *decoloratus* (red circle) , while *Rh.* (*Bo.*) *annulatus* is characterised by

four rows of denticles in the hipostome and a smooth, elongated and concave internal margin of the palp article I (red arrows).

2.5.3.2 Where *Rhipicephalus* (*Boophilus*) *microplus* does not dare (yet?)

Remarkably, *Rh.* (*Bo.*) *microplus* was not found in the study area. This suggests that the eastbound expansion in West Africa of this invasive tick species, to date found in up to five West African countries (i.e. the Ivory Coast, Benin, Burkina Faso, Mali and Togo) (Madder *et al*., 2007, 2011, 2012; Adakal *et al*., 2013) has not reached this part of Nigeria.

Its absence on the Plateau could be due to its unfavourable climate during the dry season, with very high and very low temperatures during day and night time respectively (Lee, 1972). According to a very recent distribution model based on climate suitability, the southern part of Nigeria, however, could still represent an indicated environment for the establishment of this very invasive tick (De Clercq *et al*., 2013). Moreover, a further evidence for the lack of *Rh.* (*Bo.*) *microplus* in this area is provided by the very high prevalence (41.4%) recorded for *Rh.* (*Bo.*) *decoloratus*. These two species are indeed known for competing for the expansion in a given area, with the 'cattle tick' more frequently displacing the 'Africa blue tick', as proven in several African countries such Zambia (Berkevens *et al*., 1998), Swaziland (Wedderburn *et al*., 1999), Southern Mozambique (Horak *et al.*, 2009), and South Africa (T¢nessen *et al*., 2004; Nyangiwe and Horak, 2007). It is therefore unlikely that *Rh.* (*Bo.*) *decoloratus* would survive in such great numbers, if *Rh.* (*Bo.*) *microplus* also occurred on the Plateau.

The lack of *Rh.* (*Bo.*) *microplus* is of importance not only from an epidemiological standpoint, being this tick the most competent vector of *B. bovis* (Bock *et al*., 2004), but also in terms of tick control management, as this species is known for being highly resistant to several pyrethroid and organophospate compounds (Baffi *et al*., 2008).

2.5.3.3 *Rhipicephalus guilhoni*

This study provides the first confirmed locality records for *Rh. guilhoni* in Central Nigeria. Small numbers of adults of this tick were previously collected from the cattle during the rainy season in the far north of Nigeria (Mohammed, 1974, 1977). Here, *Rh. guilhoni* was retrieved in 8 out of 9 villages and was the third most prevalent tick species.

A member of the *Rh. sanguineus* Group, this species is characterised by a more dense interstitial punctuation in the *conscutum* and a female genital aperture of a more truncated V-shape than the progenitor of its taxonomical group (Walker *et al*., 2000). It is usually found infesting cattle, sheep and camels, in steppe and savanna climatic regions (Walker *et al*., 2003). Its considerable presence on the Nigerian Plateau highlights the importance of assessing its role in infection transmission, which is so far unknown.

2.5.3.4 *Hyalomma truncatum*

The high number (n=681) of *H. truncatum* recorded reflects the seasonality of this tick in Nigeria, where it is known to peak in the late wet season (Bayer and Maina, 1984; Iwuala and Okpala, 1978). As indicated in Chapter 1, the veterinary importance of this species is related to its capacity of causing a toxic syndrome, sweating sickness, especially in young cattle (van Amstel *et al*., 1987a).

2.5.3.5 *Amblyomma variegatum*

In a study carried out over the period of one year in the neighbouring state of Kaduna, Central Nigeria, *Amblyomma variegatum* was the most prevalent species (>80% of all collected ticks) parasitizing cattle in September, followed by *Rhipicephalus* (*Bo.*) spp. and *Hyalomma* spp. (Bayer and Maina, 1984). The lower prevalence (i.e. 6%) recorded in the study with reference to *A. variegatum* could be mainly attributed to the practice of hand-picking of ticks adopted by the Fulanis, carried out up to three times a week during the wet season (Bayer and Maina, 1984). This control method mainly targets the most conspicuous *Amblyomma* adults, regarded as ‘koti’ (i.e. ‘dangerous ticks’ in Fulfulde language) by the local herdsmen, as opposed to the smaller *Rhipicephalus* and boophilid ticks that are consciously left

attached, as they are believed to be ‘miri’ (i.e. ‘less harmful’) (Maina, 1986). This operation is carried out when the animals are standing, and a number of body areas (e.g. groin, hooves, etc.) of the cattle cannot be easily reached, so that *H. truncatum* adults, that preferentially localize in the interdigital clefts and the tail switch (MacLeod, 1975; Ndhlovu *et al*., 2009) are frequently overlooked (see Figure 2.10).

Figure 2.10 – Male and (engorged) female *Hyalomma truncatum* parasitizing the interdigital area of cattle.

In addition, although it keeps the animal to some extent free from ‘tick worry’, hand removal of ticks, performed on average every ~56–72 hours (i.e. two to three days per week, respectively) (Bayer and Maina, 1984) may not help prevent the transmission of TBIs when not performed on a daily basis, as the transmission of pathogens may occur within 24-36 hours after the attachment of these arthropods to their hosts (Piesman and Spielman, 1980; Bezuidenhout, 1987; Azad and Beard, 1998). Due to their long mouthparts, *A. variegatum*, as well as *Hyalomma* ticks, can inflict serious cutaneous damage to cattle. Importantly, due to their preferential attachment to the udder and teats of cattle (MacLeod, 1975; Walker, 1996; Mattioli *et al*., 1997; Bellew and Mekonnen, 2011; Tadesse *et al*., 2012), infestation by both

these tick genera may seriously hinder the suckling of calves. As described in Chapter 1, the veterinary importance of *A. variegatum* is increased by its role as transmitter of the *Ehrlichia* (*Cowdria*) *ruminantium* (Deem *et al*., 1996a) and *Dermatophilus congolensis* (Koney *et al*., 1996) causative agents of heartwater and dermatophilosis respectively, both known to be endemic in Nigeria (Leeflang and Ilemobade, 1977a,b). Nonetheless, *A. variegatum* is also a vector of the mildly pathogenic and highly prevalent in Nigeria, *Theileria mutans* (Uilenberg *et al*., 1974; Saidu *et al*., 1984) and *Theileria velifera* (Uilenberg, 1981).

2.5.3.6 *Hyalomma rufipes*, a tick of open spaces (and sky)

The paucity (n=36) of adult *H. rufipes* collected in this study could be considered as indicative of a small population of this species, known for being widely distributed in the most arid parts of tropical Africa (Hoogstraal, 1956). Adults of *H. rufipes* are indeed usually more numerous in the early part (i.e. June-July in Nigeria) than towards the end of the rainy season (Norval, 1982). It is also possible that the altitude of the Plateau might have acted as a further limiting factor to the establishment of a *H. rufipes* population. Considering that both larvae and nymphs of this two-host tick species parasitize ground-feeding birds (Van Niekerk, 2006), it is likely that the adult specimens sampled derived from the moult of engorged nymphs brought to the study area by birds living in close contact with the herds (e.g. cattle egrets, oxpeckers, guinea fowls, etc.). Interestingly, no *H. rufipes* ticks were collected from calves (Table 2.11), suggesting that open pastures, grazed mainly by adult cattle, represent the most likely interface between cattle and these birds. Although scanty, the presence of this tick species is still of great veterinary importance as they are known to transmit *Anaplasma marginale* (Potgieter, 1979), *Theileria annulata* (Jongejan *et al*., 1983), and *Babesia occultans* (Blouin and van Renseburg, 1988) to cattle.

2.5.3.7 *Rhipicephalus simus* Group – a *taxon* to re-examine?

A relatively high number (n=239) of adult *Rh. simus* Group ticks was collected from cattle of all age groups. Of the three taxa currently ascribed to the '*simus* Group', only *Rhipicephalus muhsamae* Morel & Vassiliades (1965) was expected to be present in West Africa (Walker *et al*., 2000). However, in addition to the Group-specific punctuation pattern visible on the males' conscutum (Figure 2.11, a-b), a number of morphological features (e.g. female genital aperture and shape of adanal plates) of the specimens collected in this study appeared closely related to the East African taxon *Rhipicephalus praetextatus* Gerstäcker, 1873.

(a) **(b)**

Figure 2.11 – Adult specimens of *Rhipicephalus simus* Group: male **(a)** and female **(b)**. Bar = 1mm.

It is therefore suggested that the description of species within this taxon should be thoroughly re-examined, possibly relying also on morphometrical and molecular biology-based assays (e.g. targeting mitochondrial and nuclear targets).

On the whole, the biosystematics status of the genus *Rhipicephalus* is probably the least established within the Ixodidae family (Camicas *et al*., 1998). This is predominantly attributable to the occurrence of substantial intra-specific variability, the cause for which, historically, tick populations retrieved in different geographical areas, showing minimal morphological differences, have been considered as completely distinct species (Pegram and Walker, 1988).

It is likely that specimens identified in the present study were the same morphotype of those retrieved in the 1950s from several localities in central and Northern Nigeria, identified as *Rhipicephalus simus simus* (Unsworth, 1952).

Usually found in regions with a savanna climate, *Rh. simus* (or *Rh. simus simus*)'s distribution is believed to be restricted to Southern Africa (Pegram *et al*., 1981), where its adults preferentially parasitize cattle, never reaching high loads (Norval and Mason, 1981). Being *Rh. simus* vector of *A. centrale* (Potgieter and van Resenburgh, 1987) and *A. marginale* (Potgieter *et al*., 1983) in Southern Africa, the finding of these specimens in Nigeria may be not only of taxonomical but also of epidemiological importance.

2.5.3.8 *Rhipicephalus sanguineus* Group

Other *Rhipicephalus* species collected include *Rh. sanguineus, Rh. turanicus*, and *Rh. lunulatus*. *Rh. sanguineus* in cattle had previously been recorded elsewhere (Walker *et al*., 2000; Ouhelli and Pandey, 1982) including Nigeria (Iwuala and Okpala, 1978; James-Rugu and Jidayi, 2004; Obadiah and Shekaro, 2012; Ogo *et al*., 2012) and can be related to the presence of dogs, roaming freely within the boundaries of the villages where sampling took place, and in the vicinity of the cattle herds. This cosmopolitan three-host tick species has an association with dogs, which are the preferential host, and the human-made dwellings where they live (Dantas-Torres, 2010).

Rh. turanicus is usually more adapted to sheep and goats than cattle (Morel and Vassiliades, 1962), and this might explain the small number (n=39) of specimens collected in our study. Both *Rh. sanguineus* and *Rh. turanicus* are not known to transmit any pathogens to cattle (Dantas-Torres, 2008).

Interestingly, only one male specimen of *Rh. lunulatus* was identified. This species is characterised by very distinctive morphological features (e.g. adanal plates' shape in males; very broad U-shaped genital aperture in females) (Walker *et al*., 1988) compared to the other *Rhipicephalus* spp. ticks found in this survey. Adults of this three-host tick were previously reported in cattle in Northern Nigeria, where they were found only during the first half of the wet season (Mohammed, 1974, 1977; Unsworth, 1952). It is therefore inferable that its population dramatically diminishes towards the end of the wet season. *Rh. lunulatus* is not regarded as a hazardous tick for cattle, although it was associated with a toxicosis causing paralysis in calves in Zimbabwe (Norval and Tebele, 1983).

2.5.4 Male vs female ticks

Males constituted the majority of specimens collected for most species (i.e. *Amblyomma*, *Hyalomma* and *Rhipicephalus* spp.), with the exception of *Rh. sanguineus* and *Boophilus* spp. (Table 2.3). The male : female ratios recorded for most ticks coincided with data from previous works, with special reference to *Rh.* (*Bo.*) *decoloratus* (Kaiser *et al*., 1982, 1988; Tatchell and Easton, 1986; Bossena and Abdu, 2012), *A. variegatum* (Bellew and Mekonnen, 2012; Norval and Tebele, 1983; Kaiser *et al*., 1988; Bossena and Abdu, 2012; Asrate and Yale, 2012), *H. rufipes* (Kaiser *et al*., 1988), but not for *Rh. sanguineus* (Lorusso *et al*., 2010).

With reference to *A. variegatum* the higher proportion of males rather than females collected is attributable to the biology of this tick species, known for localizing in preferential body areas (e.g. axilla, groin, udder, scrotum) forming typical clusters including a few females clasped by several males (van Amstel *et al*., 1987b; MacLeod, 1975; see also Figures 1.7–1.8). This is due to the release of attraction-aggregation attachment pheromones (AAAP) produced only by *A. variegatum* males, attracting other unfed males and females (Norval and Rechav, 1979). This inevitably results in a concentration of more male than female specimens on the attachment sites.

The higher number of males rather than females collected for *Rhipicephalus* spp. ticks is likely due to the fact that fully engorged female ticks are more easily groomed by the animals (Yousfi-Monod, 1985) and also drop to the ground earlier to lay eggs, while males tend to remain on the host for longer periods, feeding and mating several times before dropping-off (Lorusso *et al*., 2010). The latter biological feature has been well documented for *Rh. sanguineus* (Yousfi-Monod, 1985), although in this study more females than males were collected, with a very low cumulative count (n=10).

The higher number of female rather than male boophilids collected is consistent with other studies (Tatchell and Easton, 1986) and likely reflects the relative difficulty in collecting the smaller males from hosts.

2.6 Conclusion

This study provides new information on the tick fauna composition in Nigeria and, more globally, in West Africa.

Animals were found infested with relatively high tick burdens, very likely affected by the hand-picking control adopted by the local pastoralists. Nevertheless, this did not prevent cattle from being infested from the tick species vectors of the most relevant TBIs. In fact, the finding of *Rh.* (*Bo.*) *decoloratus*, *Rh.* (*Bo.*) *annulatus*, *A. variegatum* and *H. truncatum* in all study villages is of great veterinary importance as these species are involved in the transmission of anaplasmosis, babesiosis by *Babesia bigemina* (i.e. *Boophilus* spp.); cowdriosis and dermatophilosis (i.e. *A. variegatum*); and sweating sickness (i.e. *H. truncatum*) (Young *et al.*, 1988).

Furthermore, while the indigenous cattle may better bear these tick burdens, it is very likely that exotic (i.e. *B. taurus*) or crossed (i.e. *B. indicus* x *B. taurus*) breeds, if introduced in this area, might become heavily infested with ticks thus manifesting clinically apparent TBDs, unless subjected to intensive acaricide treatment. In this case, intensive tick control would become necessary.

Therefore, further studies aiming to assess the occurrence of related TBIs in the Plateau State would be desirable, as they would also help address the possible introduction of exotic breeds in the area.

Chapter 3 – Epidemiology of cattle tick-borne haemoparasites in Central Nigeria by means of reverse line blotting

3.1 Introduction

As indicated in Chapter 1 and 2, in spite of the relatively high number of ticks on their cattle, especially in the wet season, the Fulani pastoralists do not usually employ acaricide-based means of tick control, rather relying on the manual removal of the most conspicuous specimens from certain body sites of their cattle (e.g. udder) to reduce losses of milk yields resulting from infestation (Bayer and Maina, 1984). This approach, however, does not prevent cattle from being re-infested or provide permanent relief. Conversely, as seen in Chapter 2, they remain challenged by a broad variety of tick species, most of which are vectors of TBDs-causing pathogens (Lorusso *et al*., 2013a). Several TBIs are, indeed, known for being endemic in cattle in Nigeria, including anaplasmosis (by *Anaplasma marginale* mainly), babesiosis (by *Babesia bigemina* and *Babesia bovis*), cowdriosis (by *Ehrlichia* (*Cowdria*) *ruminantium*) and theilerioris (by *Theileria mutans* and *Theileria velifera*) (Leeflang and Ilemobade, 1977a,b; Perié *et al*., 1979). With regards to *Anaplasma* infections, the literature still lacks confirmation of the presence of *A. centrale* in cattle in Northern Nigeria (Leeflang and Ilemobade, 1977b).

Oftentimes, cattle are infected by several of these haemoparasites simultaneously (Kamani *et al*., 2010), a fact from which the importance of relying on broad-spectrum specific and sensitive diagnostic tools originates (Kocan, 1995). In Nigeria, however, most investigations on cattle tick-borne haemoparasites are usually based on cytological examinations of blood smears and lymph node biopsies (Leeflang and Ilemobade, 1977a,b; Kimani *et al*., 2010) and/or serological methods (e.g. indirect fluorescent antibody test, IFAT, (IFAT, rapid card agglutination test, capillary tube-agglutination test) (Obi, 1978; Akinboade and Dipeolu, 1984; Ajay and Dipeolu, 1986). Cytological diagnosis lacks high sensitivity, being adequate only for the detection of acute infections (Friedhoff and Bose, 1994). Moreover, would mixed infections be recognized cytologically, visual discrimination between parasite species based on their morphology is usually difficult if not impossible (Figueroa *et al*., 1996; Nagore *et al*., 2004a). In addition, microscopic examination of blood smears is unreliable in case of post-acute sub-clinical/chronic TBIs (e.g. anaplasmosis, babesiosis, cowdriosis), characterized by low parasitaemia (Böse *et*

al., 1995), as well as early phase of acute infections at the onset of the disease (Nagore *et al*., 2004a). Recognizing post-acute phase sub-clinical infections is of importance, especially from an epidemiological point of view, considering that chronically infected animals serve as 'carriers' of pathogens thus acting as reservoirs for uninfected arthropods and, via the latter, other animals too (Zaugg *et al*., 1986).

Serology (e.g. IFAT) may generate negative results in case of newly active infections that have yet to stimulate seroconversion, as well as false positive results due to cross-reactions between closely related species (e.g. *Theileria* spp.) (Papadopoulos *et al*., 1996). Therefore, the use of molecular biology currently represents the most sensitive tool for diagnosing TBIs (Kocan, 1995).

In the last decade, several molecular techniques have been developed to detect tick-borne haemoparasites, in both their vertebrate or arthropod hosts, with the majority of these assays consisting of species-specific Polymerase chain reaction (PCR) assays, often with a downstream nested approach in order to obtain an enhanced sensitivity (reviewed in Sparagano *et al*., 1999; Collins *et al*., 2002; Bock *et al*., 2004; Aubry and Geale, 2011; Eremeeva, 2012). Most of the times, these tools are designed to detect single infections and are unable to diagnose co-infections by several pathogens within the same host (Sparagano *et al*., 1999; Collins *et al*., 2002; Bock *et al*., 2004; Aubry and Geale, 2011; Eremeeva, 2012). Therefore, recent research efforts had been addressed towards the development of tests enabling the simultaneous detection and differentiation of as many as possible protozoan and rickettsial parasites that could infect vertebrate hosts via tick bites. The first of which was represented by 'multiplex' PCR (mPCR), in which two or more target loci from one or more organisms are amplified using a mixture of locus-specific primer pairs within a single reaction (Edwards and Gibbs, 1994; Henegariu *et al*., 1997; Markoulatos *et al*., 2002). To date, mPCR has enabled the detection of a few bovine tick-borne microorganisms at the same time (Markoulatos *et al*., 2002), belonging to the same (i.e. *B. bigemina* and *B. bovis*) (Liu *et al*., 2014) or different genera (i.e. *A. marginale*, *B. bovis* and *Theileria annulata* or *A. marginale*, *B. bigemina* and *B. bovis*) (Figueroa *et al*., 1993; Bilgiç *et al*., 2013).

A further step towards the achievement of an 'integrated' diagnosis of bovine TBIs included the development of nucleic-acid-based assays (reviewed by Criado-

Fornelio, 2007) detecting small rRNA subunits to discriminate a few *Theileria* species (Allsopp *et al*., 1993; Bishop *et al*., 1995; Chansiri *et al*., 1999), and a multiplex loop-mediated isothermal amplification (mLAMP) for the differentiation between *B. bigemina* and *B. bovis* (Iseki *et al*., 2007).

More recently, two quantitative PCR (qPCR)-based assays were implemented, both allowing the discrimination between *B. bigemina* and *B. bovis*, one based on the amplification of a small fragment of the cytochrome b gene via the use of SYBR green (Buling *et al*., 2007), the other via a duplex TaqMan assay (Criado-Fornelio *et al*., 2009). Moreover, at the same time another qPCR employing fluorescence resonance energy transfer (FRET) probes was developed to enable the identification of several *Babesia* and *Theileria* species based on the melting temperature of their amplified fragments (Criado-Fornelio *et al*., 2009), though comparative studies showed that duplex TaqMan qPCR was more sensitive in the detection of babesias (Criado-Fornelio *et al*., 2009).

Very recently, a highly sensitive assay based on an oligonucleotide multiplex suspension microarray (Luminex® microsphere system) was developed, enabling the detection of bovine piroplasms (i.e. *Babesia* and *Theileria* spp.) (Ros-García *et al*., 2012a).

All the molecular approaches described, however, allow the identification of only a few species of the same genus or from a maximal number of three different genera (i.e. *Anaplasma*, *Babesia* and *Theileria*).

In the late 1990s, however, a polymerase chain reaction (PCR)-based reverse line blotting (RLB) method was implemented to enable the simultaneous detection of TBIs. Initially developed for detecting *Theileria* spp. and *Babesia* spp. simultaneously (Gubbels *et al*., 1999), at present the RLB allows the analysis of multiple samples (i.e. PCR products) for up to eight genera of tick-borne microorganisms, including also *Bartonella* spp. (Schouls *et al*., 1999), *Borrelia* spp. (Rijpkema *et al*., 1995; Schouls *et al*., 1999), *Ehrlichia* spp. (Schouls *et al*., 1999; Bekker *et al*., 2002; Molad *et al*., 2006), *Anaplasma* spp. (Bekker *et al*., 2002), *Hepatozoon* spp. (Matjila *et al*., 2008a) and *Rickettsia* spp. (Christova *et al*., 2003).

As further illustrated in the Appendix Section of this thesis (Section 6.1.2), to date, several RLB-based tests have been employed (Gubbels *et al*., 1999; Bekker *et*

al., 2002), alone (i.e. *Theileria*/*Babesia* spp. or *Ehrlichia/Anaplasma* spp.) (Gubbels *et al*., 2000; Ali *et al*., 2006; Salih *et al*., 2007, 2010; Muhanguzi *et al*., 2010, 2011; Yusufmia *et al*., 2010) or in combination (i.e. *Theileria*/*Babesia* spp. + *Ehrlichia/Anaplasma* spp.) (Oura *et al*., 2004; Martins *et al*., 2010; Oura *et al*., 2011; Asiimwe *et al*., 2013), in a few surveys carried out on cattle blood in SSA. This technique has yet to be applied to the monitoring of tick-borne haemoparasites in cattle in West Africa, including Nigeria, a country for which there is a lack of any published molecular survey on bovine TBIs. Furthermore, none of the RLB-based studies carried out so far in SSA, has incorporated the screening for *Rickettsia* spp. microorganisms. This genus of tick-borne Gram negative bacteria includes, amongst others, 'spotted fever group' (SFG) rickettsiae, zoonotic pathogens of increasing importance globally (Azad and Beard, 1998) as well as in SSA (Cazorla *et al*., 2008) including Nigeria (Ogo *et al*., 2012; Reye *et al*., 2012; Lorusso *et al*., 2013b). Recently the RLB method to detect *Theileria* and *Babesia* spp. was employed in conjunction with other *Anaplasma* spp.-, *Ehrlichia* spp.- and *Rickettsia* spp.-specific PCRs for the screening of a limited number (n=198) of ticks collected from cattle in Central Nigeria (i.e. Plateau and Nassarawa States), highlighting the occurrence of a rather diverse community of tick-borne pathogens of veterinary and zoonotic concern (i.e. *Anaplasma*, *Ehrlichia*, *Babesia* and *Rickettsia* spp.) in this area (Ogo *et al*., 2012).

3.1.2 The need for assessing the sensitivity of the RLB

As previously mentioned, the RLB method provides the advantageous opportunity to screen each individual sample for an array of different microorganisms at the same time. To date, however, the sensitivity of this technique has been evaluated only for a few pathogens, such as *Babesia bovis* and *Theileria annulata* (Gubbels *et al*., 1999), *Theileria parva* (Oura *et al*., 2004), *B. caballi*-like (Nagore *et al*., 2004a), and *T. separata* (Schnitter *et al*., 2004). The sensitivity of the RLB assay was also assessed for a protocol set up for the detection of *Anaplasma centrale* and *Anaplasma marginale*, targeting the *Ehrlichia*/*Anaplasma* 16S rRNA gene and employing newly designed primers (Molad *et al*., 2006). However, the sensitivity of the RLB test

employing either of the first and most commonly used primer sets for the detection of *Ehrlichia*/*Anaplasma* species (Schouls *et al*., 1999; Bekker *et al*., 2002) has not yet been assessed.

3.2 Aims

Therefore, the present study aimed to:

I. Investigate the occurrence of all possible tick-borne microorganisms infecting cattle in this area of Central Nigeria (i.e. Plateau State) where no acaricide-based vector control is usually undertaken, in spite of the relatively high burdens on cattle, during the wet season (Pullan, 1980; Bayer and Maina, 1984; Maina, 1986; Otufale and Adekoya, 2012; Lorusso *et al*., 2013a). This survey was further motivated by the finding from Chapter 2 and relied on the application of a broad spectrum-RLB, combining three different PCR reactions, enabling the detection of haemoparasites belonging to the genera *Ehrlichia* and *Anaplasma* (Schouls *et al*., 1999; Bekker *et al*., 2002), *Theileria* and *Babesia* (Georges *et al*., 2001) and *Rickettsia* spp. (Christova *et al*., 2003).

II. Assess the analytical sensitivity of the aforementioned RLB method in detecting *A. marginale*, one of the most economically relevant bovine tick-borne pathogen in West Africa (Young *et al*., 1988), known to be endemically established in Nigeria (Leeflang and Ilemobade, 1977a,b; Obi, 1978; Ajayi and Dipeolu, 1986).

In particular, the work here presented adopted an approach combining the forward primer ‘Ehr-F’ (originally ‘16S8FE’) designed by Schouls *et al.* (1999) and the reverse one ‘Ehr-R’ (originally ‘BGA1B-new’) designed by Bekker *et al.* (2002), as an update of the ‘BGA1B’ initially designed by Schouls *et al.* (1999) (see Table 3.2).

Moreover, this study aimed to assess, whether bovine DNA does play a role interfering with *A. marginale* detectability by means of RLB using the protocol described above.

Methodologies and results illustrated in this chapter, as well as in Chapter 4, represent the outcome of a four-year long collaboration between the Welburn Research Group, Division of Pathway Medicine, the University of Edinburgh, UK, the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Plateau State, Nigeria, and the Utrecht Centre for Tick-borne Diseases (UCTD), Utrecht University, The Netherlands.

3.3 Materials and Methods

3.3.1 Study area

The study was conducted in nine villages belonging to three neighbouring local government areas (LGAs), namely Bokkos, Mangu and Pankshin, in the central part of Plateau State, Nigeria (9.14´–9.59´ N, 8.84´–9.38´ E) (Figure 3.1). The study area falls within the North Savanna vegetation zone, in the sub-humid region of Nigeria, where the dry season generally extends from November to April, and the wet season from April-May to October, with most (~80%) of the rains occurring between June and September (Odumodu, 1983).

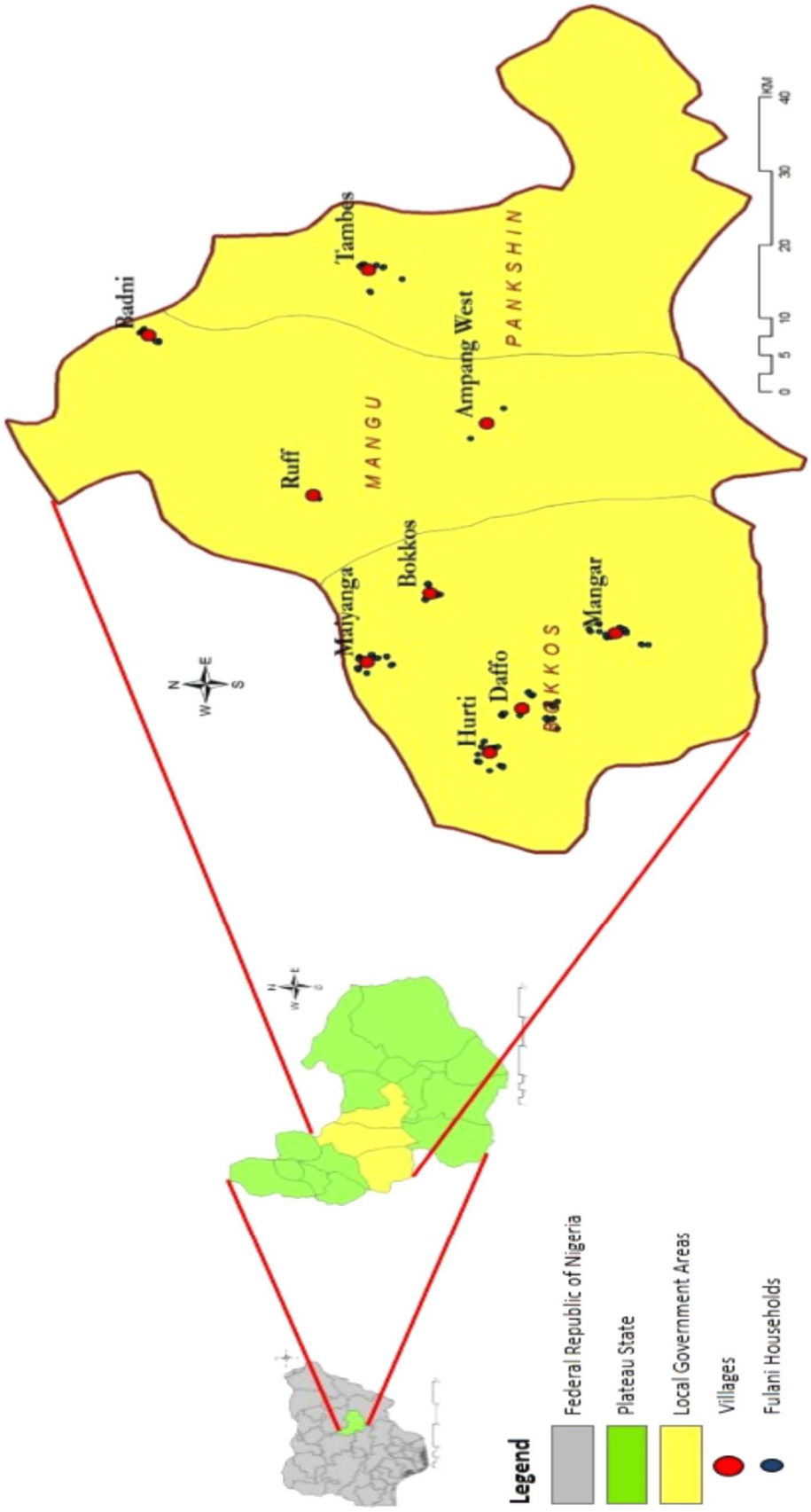

Figure 3.1 – Map of the study area.

All cattle reared in the area are of indigenous (*Bos indicus*) genotype, of which approximately 80% belonging to the White Fulani breed, and a smaller number are of either Bunaji or White Fulani x Bunaji crossbreeds. Cattle are grazed on communal pastures year-round according to the traditional Fulani herding system. Other livestock reared in the area include goats, sheep, pigs and poultry. In all villages, dogs are kept as guards of the households.

3.3.2 Ethics statement

This study was carried out with the approval of the Ethical Committee on Animal Use and Care at the Nigerian Institute for Trypanosomiasis (NITR) research, Vom, Nigeria. All cattle enrolled in the study were selected and sampled with the agreement of their owners and chief of the Fulani villages. Animals were handled humanely, being restrained with the help of their owners for the purpose of sampling.

3.3.3 Sample collection

704 indigenous (*B. indicus*) cattle of various sex and age (i.e. 43 calves, 184 juveniles and 477 adults) were randomly selected in nine villages, identified as a subset of a previously conducted cluster sampling (Majekodunmi *et al*., 2013) for being representative of the agro-ecological zone, the cattle population and management in the Plateau State, Central Nigeria (Table 3.1). Age of the animals was estimated on the basis of the dentition score method developed for zebu cattle under a low plane of nutrition (Kikule, 1953) and on information provided by their owners. Once quantified, each animal's age was recorded either as 'calf' (0–6 months), 'juvenile' (6–24 months), or 'adult' (older than 24 months).

Table 3.1 – Cattle screened grouped according to the village of sampling.

Village Name	Total cattle population	Animals sampled			
		Calves	**Juveniles**	**Adults**	**Totals**
Ampang West	790	0	33	47	**80**
Badni	383	4	20	56	**80**
Bokkos	2,142	6	25	49	**80**
Daffo	2,933	4	17	51	**72**
Hurti	1,011	2	9	69	**80**
Maiyanga	2,543	6	32	42	**80**
Mangar	1,373	5	29	46	**80**
Ruff	154	5	18	49	**72**
Tambes	854	11	1	68	**80**
Total	**12,183**	**43**	**184**	**477**	**704**

Whole blood was collected from each animal by jugular venipuncture; approximately 100 µl of it were applied directly onto Flinders Technology Associates (FTA™) cards (Whatman BioScience Ltd, Cambridge, UK) (Figures 3.2–3.3). After being allowed to air-dry over night at room temperature, all samples were placed in foil pouches with a silica desiccant and mailed to the University of Edinburgh for molecular processing.

Figure 3.2 – Blood sampling of a juvenile White Fulani cow via jugular venipuncture (© Marie Julie Ducrotoy).

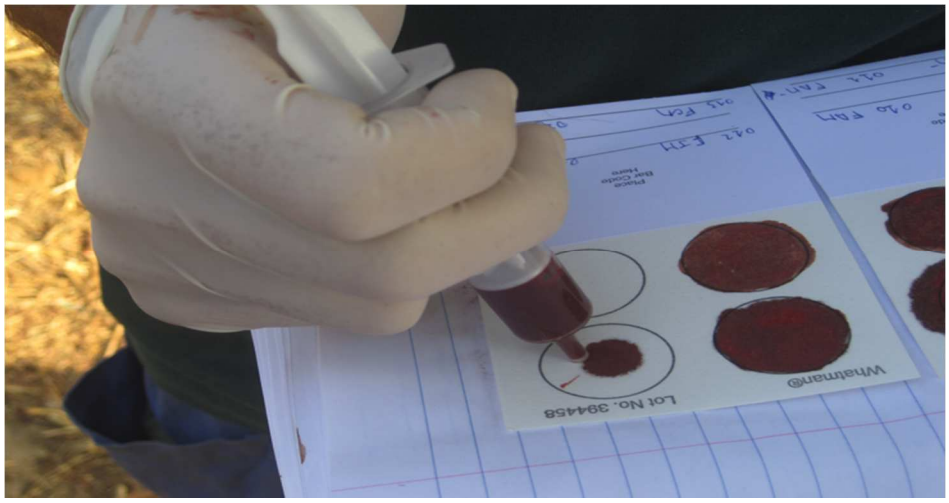

Figure 3.3 – Spotting of ~100 µl of whole blood on to a single circle of FTA™ card matrix.

3.3.4 Molecular proceedings

Once in the laboratory, each sample was subjected to a molecular protocol consisting of DNA extraction and elution, three simultaneous PCR amplifications followed by RLB. The RLB hybridization assay is a versatile tool that can be used to detect and differentiate tick-borne microorganisms in several types of biological samples including blood, tissues (e.g. lymph node biopsies, brain smears, etc.) and/or in arthropods (UCTD, 2013). RLB is performed next to PCR, by applying the products of the first amplification(s), diluted in a buffer, onto a blotting membrane.

The essence of the technique is the hybridization of fragments of PCR products with specific dozens-nucleotide long probes previously immobilized (i.e. covalently linked) on the aforementioned membrane.

3.3.4.1 DNA extraction and elution

Once in the laboratory, five three mm-circular portions of each sample-saturated matrix of each FTA™ card (equivalent to a volume of 13.2 µl), were punched using a Harris Micro Punch™ (Whatman BioScience Ltd, Cambridge, UK) and placed in a sterile 1.5 ml eppendorf tube. To increase the chances of detecting haemoparasites' DNA (thus avoiding false negative results), punching was carried out on five different areas of each sample's circle (e.g. referring to each circle as a clock: one punch was performed at 12 o' clock, another one at 3, one at 6, one at 9 and one in the center of the circle; see Figure 3.4).

Figure 3.4 – Picture of an FTA™ card
showing the remains of a sample (#35) after the punching of five dics.
The technique enables multiple utilizations of the initial sample collected**.**

Between each sample, 15 blank FTA™ discs were also punched to avoid carry-over contamination. Negative controls for the DNA extraction and elution

phases were represented by five blank (white) FTA™ discs, punched before the first ('W_0'), the 20th ('W_1') and the 40th sample ('W_2'). During a preliminary pilot test negative controls were employed between each of 20 samples, after 15 blank punches, resulting all negative at both PCR and RLB (data not shown).

After the 40th sample of a batch, the Harris Micro Punch™ was rinsed with concentrate bleach and 70% ethanol, before being wiped and UV-ed for 30 minutes. After being punched, discs of each sample were then washed twice for 15 minutes in 1ml of FTA™ purification reagent (Whatman BioScience Ltd, Cambridge, UK) to remove any PCR inhibitors from the sample, and rinsed twice for 15 minutes in 1ml of 1x Tris-EDTA buffer (Sigma Aldrich, Dorset, UK) to remove traces of FTA™ purification reagent. Each test sample (i.e. 5 discs) was then carefully transferred to a sterile 0.2 ml PCR tube and allowed to air-dry at 37ºC for 40 minutes. Afterwards, each samples' DNA was eluted by adding 100 µl of 5% (w/v) Chelex® 100 (Sigma-Aldrich Ltd, UK) solution to each PCR tube and incubating at 90°C for 30 minutes in a thermal cycler.

3.3.4.2 PCR

After elution, each sample was subjected to three PCR amplifications targeting a 460–540 bp long fragment from the V4 hypervariable region of the 18S ribosomal RNA (rRNA) gene for *Theileria* and *Babesia* spp. (Georges *et al*., 2001), a 460–520 bp long fragment from the V1 hypervariable region of the 16S SSU rRNA gene for *Ehrlichia* and *Anaplasma* spp. (Schouls *et al*., 1999; Bekker *et al*., 2002), and a 350–400 bp variable region in the 16S rRNA gene for *Rickettsia* spp. (Christova *et al*., 2003) (see Table 3.2).

One of the two primers employed for each set (i.e. the reverse one, as in most cases, see Table 3.2) was biotin-conjugated at the 5' end, to enable the further binding of the PCR products, once hybridized to probes on the blotting membrane, with a streptavidin ligand employed within the RLB protocol (see Section 3.3.4.3.4, Figure 3.10).

Moreover, in order to enhance their capacity to bind to different target DNA of microorganism belonging to genera with rather high variability (e.g. *Ehrlichia* and *Anaplasma* spp.), the PCR primers employed, were designed with mixed (also

known as 'degenerate') bases in their nucleotide sequence [i.e. A/C (M) in primer 16S8FE (Schouls *et al*., 1999), C/T (Y) in BGA1B-new (Bekker *et al*., 2002), see also Table 3.2]. During their manufacturing process, equal amounts of each base were delivered to the primers' sequence by the synthesizer (EMBO and ICTTD, 2003). The same principle was applied to some nucleotide probes (e.g. *Anaplasma bovis*-, *Theileria*/*Babesia* catch-all-, *Theileria buffeli*-specific probes), chosen for the RLB membrane used (see Table 3.5).

Table 3.2 – Primers used in the PCR for the *Ehrlichia*/*Anaplasma* spp. (Ehr-F / Ehr-R), *Theileria*/*Babesia* spp. (RLB-F2 / RLB-R2), *Rickettsia* spp. (Rick-F / Rick-R) simultaneous amplification.

Primer	Sequence (5'– 3')	Orientation	Tm* (°C)	Reference
Ehr-F (a.k.a. 16S8FE)	GGAATTCAGAGTTGGATC(A/C)TGG (C/T)TCAG	+	61.0	Schouls *et al*. (1999)
Ehr-R (a.k.a. BGA1B-new)	Biotin-CGGGATCCCGAGTTTGCCGGGACTT (C/T)TTCT	-	69.5	Bekker *et al*. (2002)
RLB-F2	GACACAGGGAGGTAGTGACAAG	+	57.9	Georges *et al*. (2001)
RLB-R2	Biotin-CTAAGAATTTCACCTCTGACAGT	-	53.7	
Rick-F	GAACGCTATCGGTATGCTTAACAC A	+	66.9	Christova *et al*. (2003)
Rick-R	Biotin-CATCACTCACTCGGTATTGCTGGA	-	69.4	

*Tm=melting temperature.

Each PCR was carried out on a total volume of 25 µl, using 5 µl of 5x Phire reaction buffer (Thermo Scientific, USA), 0.5 µl of 10 mM dNTPs (Rovalab GmbH, Germany), 0.5 µl of 20 pmol/µl of each forward and reverse primer (Integrated DNA Technologies, Inc., USA), 0.25 units of Phire Hot Start II DNA polymerase (Thermo Scientific, USA), 15.875 µl of water, and 2.5 µl of template DNA (summarized in Table 3.3).

Table 3.3 – Master mix composition with the working volume for one 5 µl sample (Total volume of reaction: 25 µl).

Reagent	Composition	Working Volume
Sterile PCR Water	Dnase/Rnase-free MilliQ water	15.875 µl
10x NH_4 Buffer	160mM $(NH_4)_2SO_4$, 670mM Tris-HCI (pH8.8 at 25°C) and stabilizers	5.0 µl
Nucleotides	dATP, dCTP, dGTP, dUTP, 10mM solution	0.5 µl
Forwad Primer	Ehr-F / RLB-F2 / Rick-F (20 pmol/µl) in MilliQ water	0.5 µl
Reverse Primer	Ehr-R / RLB-R2 / Rick-R (20 pmol/µl) in MilliQ water	0.5 µl
Phire Hot Start II DNA Polymerase	(2 units/µl)	0.125 µl

Positive controls included 2.5 µl of DNA from *Theileria parva* (Accession No. KJ095110), *Ehrlichia canis* (Accession No. KJ095115), and rickettsial DNA >98% similar to *Rickettsia africae* (Accession No. JX101606) (Alberdi *et al*., 2012), for the three aforementioned PCRs respectively. Negative controls consisted of 2.5 µl of water and 5% (w/v) Chelex® 100 (Sigma-Aldrich Ltd, UK)-eluted blank white paper.

To minimize non-specific annealing, a touchdown PCR program was used. The fact that the primers employed had rather close melting temperatures (see Table 3.2), allowed the use of the same programme for all three PCRs (schematised in Table 3.4). DNA amplification was carried out in a Dyad Peltier thermal cycler© (MJ Research Inc., USA), following the programme illustrated in Table 3.4.

Table 3.4 – PCR conditions for all three primer sets employed.

PCR step	Temperature	Duration (seconds)	Number of cycles
Initial Denaturation & Hot-start polymerase activation	98°C	30s	1
Denaturation	98°C	5s	10*
Annealing	67–57°C	5s	
Extension	72°C	7s	
Denaturation	98°C	5s	40
Annealing	57°C	5s	
Extension	72°C	7s	
Final extension	72°C	60s	1

*Temperature decreasing of 1°C at each cycle.

After PCR, positive and negative controls were subjected to an electrophoretic run in 1.5% (w/v) agarose gel stained with GelRed© (Biotium, USA), using an exACT™ Gene Low Range Plus (100–2000 bp) DNA ladder (Fisher Scientific UK Ltd, Loughborough, UK) in order to verify the correct execution of the amplification (positive controls showing bands of the expected size) in absence of contamination (negative controls showing no bands; see Figure 3.5).

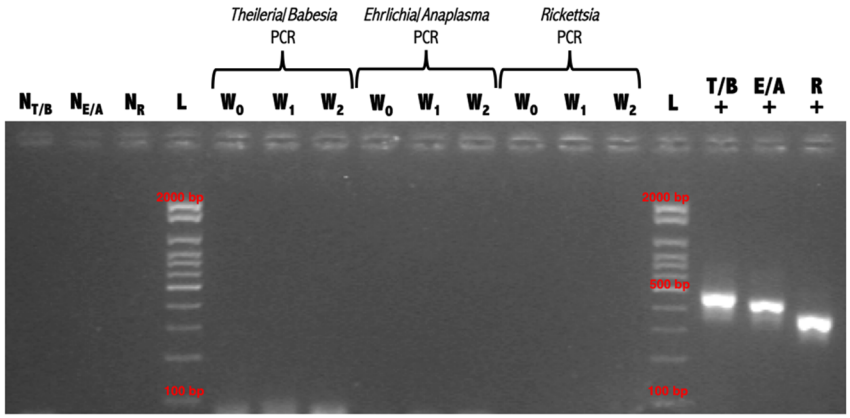

Figure 3.5 – Visualization of extraction and PCR controls under UV light after electrophoretic run in 1.5% w/v agarose gel.

[$N_{T/B}$ = negative *Theileria*/*Babesia* PCR control; $N_{E/A}$ = negative *Ehrlichia*/*Anaplasma* PCR control; N_R = negative *Rickettsia* PCR control; W_0, W_1, W_2 = blank FTA™ card controls; T/B+ = *Theileria*/*Babesia* positive control (i.e. *T. parva*, 433 bp); E/A+ = *Ehrlichia*/*Anaplasma* positive control (i.e. *Ehrlichia canis*, 426 bp); R+ = *Rickettsia* positive control (i.e. *Rickettsia africae*-like 16S, 365 bp)].

3.3.4.3 Reverse line blotting (RLB)

3.3.4.3.1 Design of the membrane

'Catch-all' and species-specific oligonucleotide probes containing a N-terminal *N*-(trifluoroacetamidohexyl-cyanoethyle, *N*,*N*-diisopropyl phosphoramidite [TFA])-C6 amino linker (Isogen, The Netherlands) were covalently linked onto a Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.) as described by Gubbels *et al.* (1999). Catch-all probes were included to ensure that the hybridization of positive samples took place correctly (i.e. all samples turning positive for one of more species are expected to be positive for the 'catch-all' probe of their respective genus), but also to indicate the presence of an unknown species or strain of a certain parasite or the presence of a known species, for which a specific oligonucleotide probe was not included in the assay.

For each catch-all and species-specific probe employed, the amount of oligonucleotide applied on the membrane was optimized to 400 µM, enabling the visualization, in presence of the same amount of DNA, of equally intense signals. In order to obtain the desired aforementioned working concentration (i.e. 400 µM), oligonucleotide probes were diluted in 150 µl of $NaHCO_3$ at a pH of 8.4. Prepared dilutions were then applied on the blotting membrane, previously 'activated' via a 10 minute-long incubation step in 16% 1-(3-Dimethylaminopropyl)-ethylcarbodiimide hydrochloride (EDAC) at room temperature (UCTD, 2013), through the use of a Miniblotter MN45 (Immunetics, MA, USA). Afterwards, the membrane was left to incubate for one minute, after which the loaded volumes of $NaHCO_3$ were removed by aspiration, in the same order as they were applied (UCTD, 2013). The biodyne membrane was then washed for 8–10 minutes at room temperature in a tray containing 100 ml of 100 mM Sodium hydoxide (NaOH) in order to lose its EDAC-

due reactiveness (UCTD, 2013). A washing step in a buffer (2x SSPE/0.1% SDS) at 60ºC for 10 minutes concluded the process of membrane preparation.

The sequences of the nucleotide probes employed in the study described in this chapter are reported in Table 3.5, enabling the simultaneous screening of each sample for up to five different genera (i.e. *Ehrlichia*, *Anaplasma*, *Theileria*, *Babesia* and *Rickettsia*) and 12 species of tick-borne microorganisms. The choice of genus- and species-specific probes employed was based on the existing literature on bovine TBIs in West Africa as well as the field experience of veterinarians and scientists from the Nigerian Instititute of Trypanosomiasis Research (NITR) and the Parasitology group of the Nigerian Veterinary Research Institute (NVRI). Moreover, the inclusion in the membrane of a probe specific for *Theileria parva*, whose distribution does not include West Africa (Norval *et al*., 1992), enabled the use of the same positive control in both PCR and RLB for the *Theileria*/*Babesia* spp. 18S amplification.

Table 3.5 – Catch-all and species-specific oligonucleotide probes fixed on the RLB membranes employed (listed in the same order as they were loaded).

	Tick-borne Microorganism's Genera/Species	**Probe Sequence (from 5'–3')**	**Tm (ºC)**	**Reference**
1	*Ehrlichia/ Anaplasma* catch-all	GGGGGAAAGATTTATCGCTA	58	Bekker *et al*. (2002)
2	*Anaplasma bovis*	GTAGCTTGCTATG(A/G)GAACA	56–58	Georges *et al*. (2001)
3	*Anaplasma centrale*	TCGAACGGACCATACGC	61	Bekker *et al*. (2002)
4	*Anaplasma marginale*	GACCGTATACGCAGCTTG	59	Bekker *et al*. (2002)
5	*Ehrlichia ruminantium*	AGTATCTGTTAGTGGCAG	54	Bekker *et al*. (2002)
6	*Ehrlichia* sp. Omatjenne	CGGATTTTTATCATAGCTTGC	57	Bekker *et al*. (2002)
7	*Theileria/Babesia* catch-all	TAATGGTTAATAGGA(A/G)C(A/G) GTTG	55–59	Matjila *et al*. (2008a)
8	*Babesia* catch-all 1	ATTAGAGTGTTTCAAGCAGAC	57	Nijhof (unpublished)
9	*Babesia* catch-all 2	ACTAGAGTGTTTCAAACAGGC	60	Nijhof (unpublished)
10	*Babesia bigemina*	CGTTTTTTCCCTTTTGTTGG	58	Gubbels *et al*. (1999)
11	*Babesia bovis*	CAGGTTTCGCCTGTATAATTGAG	61	Gubbels *et al*. (1999)

	Tick-borne Microorganism's Genera/Species	Probe Sequence (from 5'–3')	Tm (ºC)	Reference
12	*Theileria annulata*	CCTCTGGGGTCTGTGCA	62	Georges *et al*. (2001)
13	*Theileria buffeli*	GGCTTATTTCGG(A/T)TTGATTTT	56–57	Gubbels *et al*. (1999)
14	*Theileria mutans*	CTTGCGTCTCCGAATGTT	59	Gubbels *et al*. (1999)
15	*Theileria parva*	GGACGGAGTTCGCTTTG	60	Nijhof *et al*. (2003)
16	*Theileria taurotragi*	TCTTGGCACGTGGCTTTT	62	Gubbels *et al*. (1999)
17	*Theileria velifera*	CCTATTCTCCTTTACGAGT	54	Gubbels *et al*. (1999)
18	*Rickettsia* catch-all	TTTAGAAATAAAAGCTAATACCG	54	Christova *et al*. (2003)

*Tm = melting temperature.

Catch-all and species-specific oligonucleotide probes employed in the RLB assay implemented for this study had rather close melting temperatures (ranging between 54–62ºC), so that all of them could hybridize with their respective complementary target DNA fragments while exposed to the temperature of incubation of 42ºC.

To help identify the order in which the probes were fixed on the blotting membrane, thus orient the loading of the samples, diluted Indian ink was applied onto two parallel edges of the membrane, in parallel to the direction of probe loading, and the bottom right corner of the Byodine membrane was also cut blunt (see Figure 3.6).

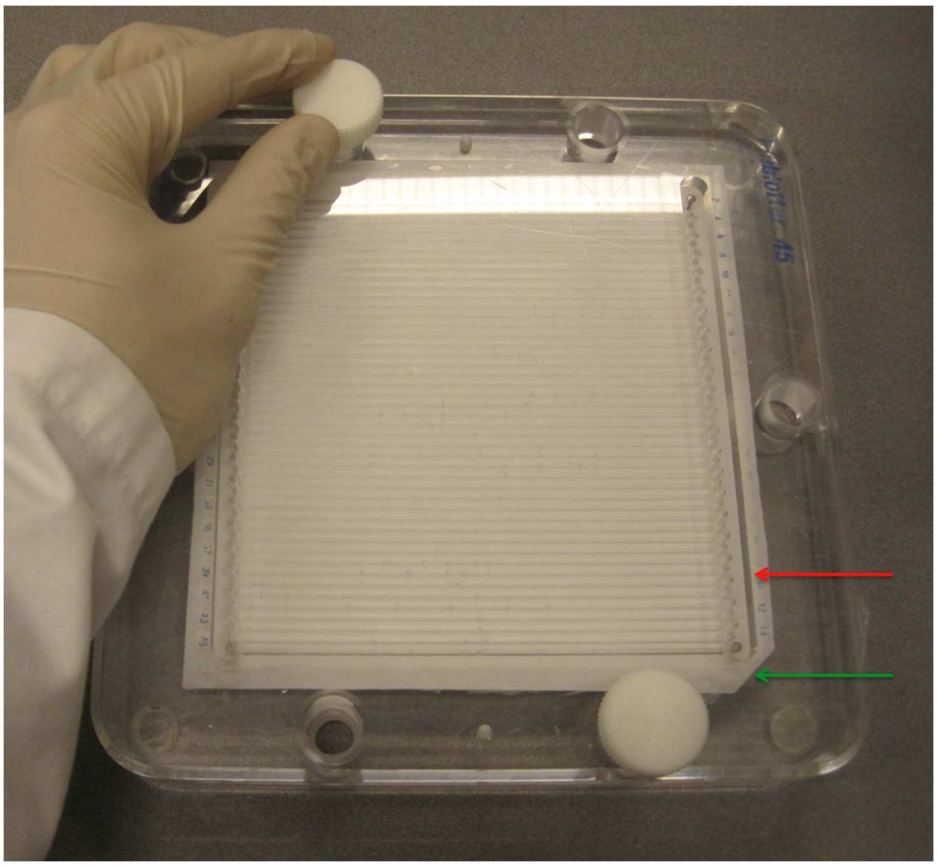

Figure 3.6 – Biodyne membrane being secured within a Miniblotter MN45 (Immunetics, MA, USA) before the loading of samples.

Note the blunt bottom right corner (green arrow) and the Indian ink-black line (red arrow) indicating the orientation according to which oligonucleotide probes were previously fixed on the membrane. Samples will be loaded perpendicularly to them (see lanes #1–45).

3.3.4.3.2 Sample preparation

Following membrane preparation and the PCR amplification, RLB was perfomed. To do so, 10 µl of all three (i.e. *Ehrlichia*/*Anaplasma* spp., *Theileria*/*Babesia* spp. and *Rickettsia* spp.) PCR products obtained from each individual DNA sample were mixed with 130 µl of 2x SSPE/0.1% SDS buffer to a total volume of 160 µl. Positive controls were prepared adding 10 µl of the respective PCR product in 150 µl of 2x SSPE/0.1% SDS. The same procedure was followed for the negative PCR controls, with 10 µl of each product being diluted into 150 µl of the 2x SSPE/0.1% SDS. For the extraction/elution negative controls, 10 µl of each of the three different (i.e. *Ehrlichia*/*Anaplasma* spp., *Theileria*/*Babesia* spp. and *Rickettsia* spp.) PCR products originating from the same control (e.g. 'W_0' or

'W_1' or 'W_2') were mixed into 130 µl of 2x SSPE/0.1% SDS before being loaded on the membrane. Therefore, each RLB contained a total of nine controls, including: three positive controls, three negative 'PCR' controls and three negative 'extraction/elution' controls (see Figure 3.11a). Inevitably, all PCR negative controls functioned as negative control of the RLB reaction too.

Before being loaded on the blotter, the PCR products diluted in the buffer (i.e. 2x SSPE/0.1% SDS) were heated up to 100ºC for 10 minutes, long enough for DNA to denature and therefore become single-stranded (Gubbels *et al.*, 1999). Afterwards, samples were cooled rapidly on ice (see Figure 3.7). After cooling down, samples were centrifuged for 30s at 11,000 x G in a pre-chilled centrifuge at 4°C. From this step until the actual loading on the blotting membrane, samples were kept on ice, in order to prevent the DNA from reannealing (UCTD, 2013) (Figure 3.7).

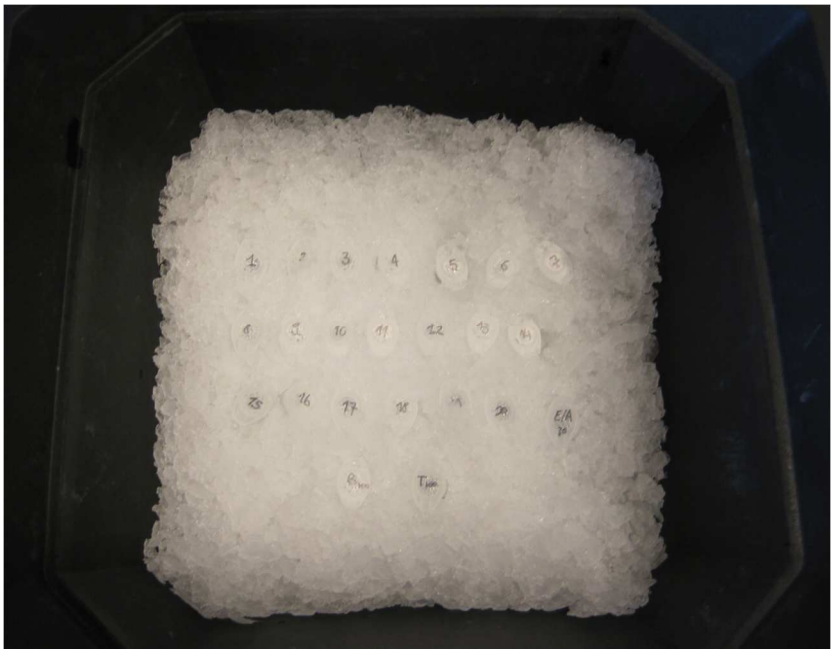

Figure 3.7 – Samples in 1.5 ml eppendorf tubes kept in ice after denaturation at 100ºC for 10 minutes, to prevent DNA from double stranding, prior to loading on the blotting membrane.

3.3.4.3.3 Membrane loading

Hundred-and-sixty µl of each sample and control preparation was loaded onto a Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.), using a Miniblotter MN45 (Immunetics, MA, USA). Samples were applied on the membrane

in a perpendicular direction to the oligonucleotide probes' orientation (Figure 3.8), to ensure that the 'microorganism DNA strand/oligonucleotide sequence' hybridization could occur at the cross-sections of the line containing the probe and the line containing the dilutedPCR product (Figure 3.9).

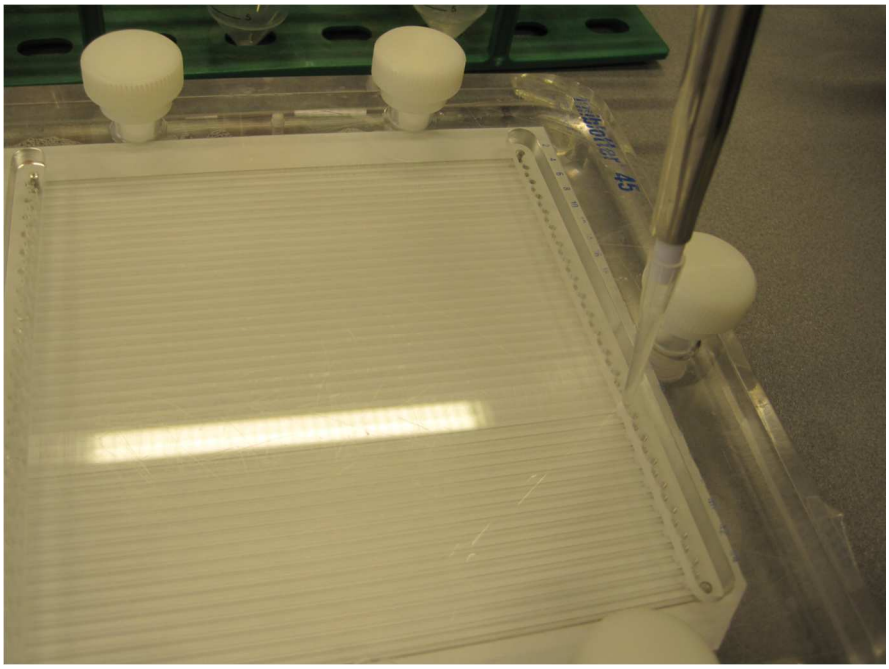

Figure 3.8 – Sample loading on a Miniblotter MN45 (Immunetics, MA, USA).

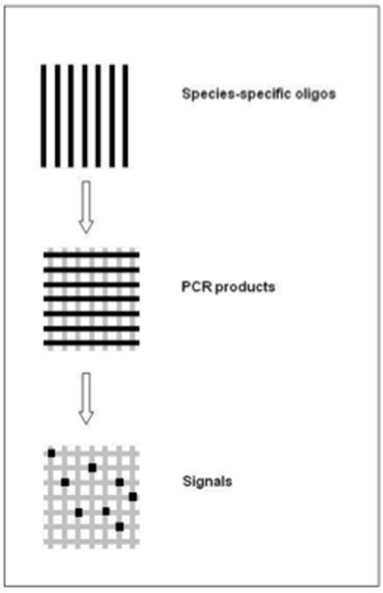

Figure 3.9 – Principle of the RLB assay (from EMBO and ICTTD, 2003).

3.3.4.3.4 RLB hybridization

After loading them on the RLB membrane, samples were allowed to hybridize for one hour at 42°C (see also Figure 3.10).

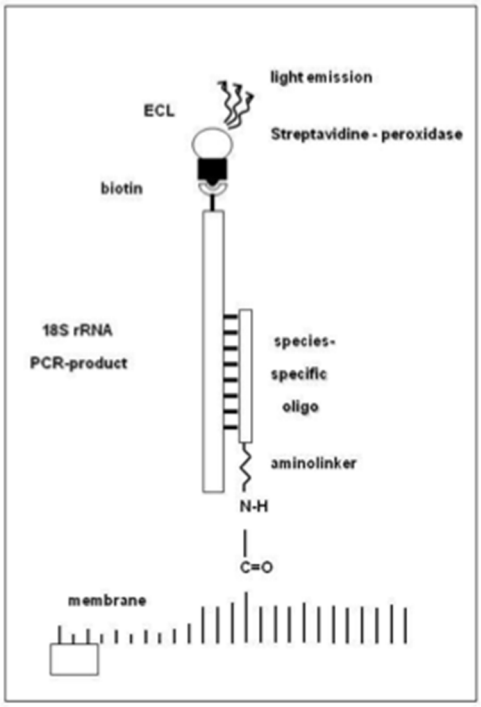

Figure 3.10 – Principle of the RLB hybridization (from EMBO and ICTTD, 2003).
The figure provides a schematic representation of the mechanism according to which the probe-DNA fragment interaction is revealed via the bond between the biotin-labelled (5') DNA and streptavidin. The latter ligand, in turn, bears an enzymatic label (i.e. peroxidase) reacting with the ECL substrate, thus producing a chemiluminiscent signal that can be detected via impression of an x-ray film.

Afterwards, samples were removed using aspiration and stringent washing was carried out to remove unbound PCR products according to Gubbels *et al.* (1999) with the modification that the first 2 washing steps were performed at 50°C for 10 minutes to remove false annealed PCR products.

3.3.4.3.5 X-ray development and results visualization

After stringent washing, hybridized PCR products were detected by chemiluminescence reactions, using ECL reagents (Amersham, UK) after the labeling of biotin with streptavidin horseradish peroxidase. Finally reactions were visualized using ECL hyperfilm films (Amersham, UK), exposed to the membrane

for 10 minutes in a dark room (UCTD, 2013). Developing of the ECL hyperfilm was carried out with the use of an x-ray developer (Protec GmbH, Germany).

After correct exposure and development, spots occur at the sites where the catch-all and species-specific oligonucleotide probes and PCR products hybridized (see Figures 3.9 and 3.11a,b). Finally, the identity of the microorganism(s) present in the samples processed was confirmed using a grid bearing the order of probes according to how they were loaded on the membrane, printed on a A4 white sheet juxtaposed to the developed x-ray film (UCTD, 2013; see also Figure 3.11a,b).

3.3.4.3.6 Membrane stripping and storing between uses

After RLB, the byodine membrane was 'stripped' in order to remove all the DNA bound to its probes. Stripping was carried out washing the membrane twice, under gentle shaking, at 70–80ºC with a pre-heated saponin buffer (10% SDS) applied each time for half an hour.

After stripping the membrane was incubated in 20 mM Ethylenediaminetetraacetic acid (EDTA) for 15 minutes at room temperature, to prevent fungal growth in case of long storage (UCTD, 2013). Between several uses membranes were stored at 4ºC within sealing bags filled with a modest volume (<100 ml) of EDTA. This would enable the re-use of the same membrane, for a total number of at least 20 times (Gubbels *et al*., 1999; Sparagano *et al*., 2000).

3.3.4.4 Sequencing

To further ascertain species identity, samples hybridizing only with a catch-all probe were subjected to DNA purification using the QIAquick Gel Extraction Kit (Qiagen GmbH) and single read sequencing via a Sanger ABI 3730xl (GATC Biotech, Germany). Initial sequence inspection, cleaning, and alignment were conducted using Bioedit (Hall, 1999). Sequences were then identified with the use of the Basic Local Alignment Search Tool (BLAST) (NCBI Blastn). Selected sequences amongst those obtained were deposited in GenBank on 3 December 2013. Submission was successful and accession numbers are currently being generated at NCBI.

3.3.5 Statistical analysis

Prevalence of infected animals, single and multiple infections, and of each tick-borne microorganism were calculated in the R software (http://www.R-project.org) with the 'survey' package, using the exact binomial 95% confidence interval (CI) and the reciprocal of the sample size.

Chi-square test in the WinPepi software was used to test the null hypothesis for significant difference between age classes (i.e. calves, juveniles, and adults) with regards to overall and individual haemoparasite infection.

Villages were compared for overall infection rates (i.e. number of infected versus non-infected animals) using the likelihood-ratio test followed by Bonferroni correction, with the WinPepi software.

Frequency of combinations of co-infective tick-borne haemoparasites were calculated by normal counts according to age classes. Moreover the statistical likelihood of all possible infection patterns detectable in this study was assessed through the association screening approach as described in Vaumourin *et al*. (2014), considering the three age classes identified (i.e. calves, juveniles and adults) altogether. Briefly, the association screening approach is a test based on a simulated theoretical distribution of a statistic and its associated confidence interval, under the null hypothesis H0 that infection patterns (i.e. parasite associations or single infections) are random. In the case of this study, the occurrence (i.e. counts) of all possible combination of parasites or single infections, was theoretically simulated, with each infection pattern (either single or type of multiple infections) being exclusive of one another. The 'envelope ()' function from the 'boot' package in the R software (www.R-project.org) was used to estimate the 95% confidence envelope for the combination count distribution profile that includes all possible infection patterns. A global test based on the 95% confidence envelope was first run. When H0 was rejected, the local tests based on the number of possible parasite combinations confidence intervals were performed.

For all statistical tests employed, p values lower than 0.05 were considered as statistically significant.

3.3.6 Sensitivity assessment of the RLB hybridization for the detection of *Anaplasma marginale*

DNA obtained from the whole blood of a calf (#4291) experimentally infected within the facility of Utrecht University, The Netherlands, with an *Anaplasma marginale* isolate from Zaria, Nigeria (Zivkovic *et al*., 2007), was used to assess analytically the sensitivity of the RLB method. A PCR product of approximately 450 bp obtained from this stabilate using the *Ehrlichia*/*Anaplasma* 16S primer set (Bekker *et al*., 2002) without the biotin label in the Ehr-R, was purified and cloned using the CloneJET PCR Cloning Kit (Thermo Scientific, USA) following the instructions of the manufacturer. After the ligation and the transformation of *Escherichia coli* DH5α, cloned plasmids were purified using the GeneJET plasmid Miniprep kit (Thermo Scientific, USA) from several overnight cultures and subjected to PCR to confirm insert length. Suspected positive clones were further purified and send in for sequencing for confirmation of species identity using BLAST (NCBI Blastn). Sequence analysis revealed 100% identity with *A. marginale* 16S rRNA gene [GenBank Accession No. KJ095114]. Three purified clones from three different PCRs were used to prepare two series of 15 ten-fold dilutions each, starting from 4.3×10^{10} copies/µl (initial mean concentration: ~150 ng/µl) to 4.3×10^{-5} copies/µl. In order to assess whether any host DNA may interfere with the detectability of the chosen pathogen, for each clone, two dilution series were prepared adding 10 µl of purified plasmid elution (4.3×10^{10} copies/µl) into 90 µl of water and into 90 µl of control bovine genomic DNA (BioChain®, USA) adjusted with a 1x TE buffer to an average concentration of 30 ng/µl (Foley *et al*., 2011), respectively. For each dilution series, all 10-fold serial dilution preparations were subjected to PCR using the aforementioned primer set (Schouls *et al*., 1999; Bekker *et al*., 2002), targeting a common region in the 16S rRNA gene for *Anaplasma* and *Ehrlichia* spp. (see also Section 3.3.4.2). Afterwards, 10 µl of each PCR product was processed and visualized by RLB using the aforementioned method.

3.4 Results

3.4.1 RLB development

An example of representative sets results is given by Figure 3.11 (a–b).

For *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* spp., all species-specific spots distinguishable at naked eye and accompanied by a discernable (usually, but not necessarily, more intense) signal in correspondence of their respective catch-all probe, were considered as 'positive' results. Irrespective of their intensity, catch-all only positive signals (i.e. *Ehrlichia*/*Anaplasma* spp., *Theileria*/*Babesia* spp. and *Rickettsia* spp.) were considered as positive results when their outline was discernable.

'Doubtful' either species-specific or catch-all results included those particularly faint signals that could not be discriminated after applying the developed x-ray film on a white A4-sized paper sheet on which the reading grid had been printed (see Figure 3.11, a–b). PCR amplicons of these samples were therefore re-run on a successive RLB employed the same membrane after correctly stripping it. If the same type of signals were obtained, the samples were considered as 'positive'. In the studies described in Chapter 3 and 4 of this thesis, re-testing was needed for a few (n<10) *Theileria*/*Babesia* spp. positive samples.

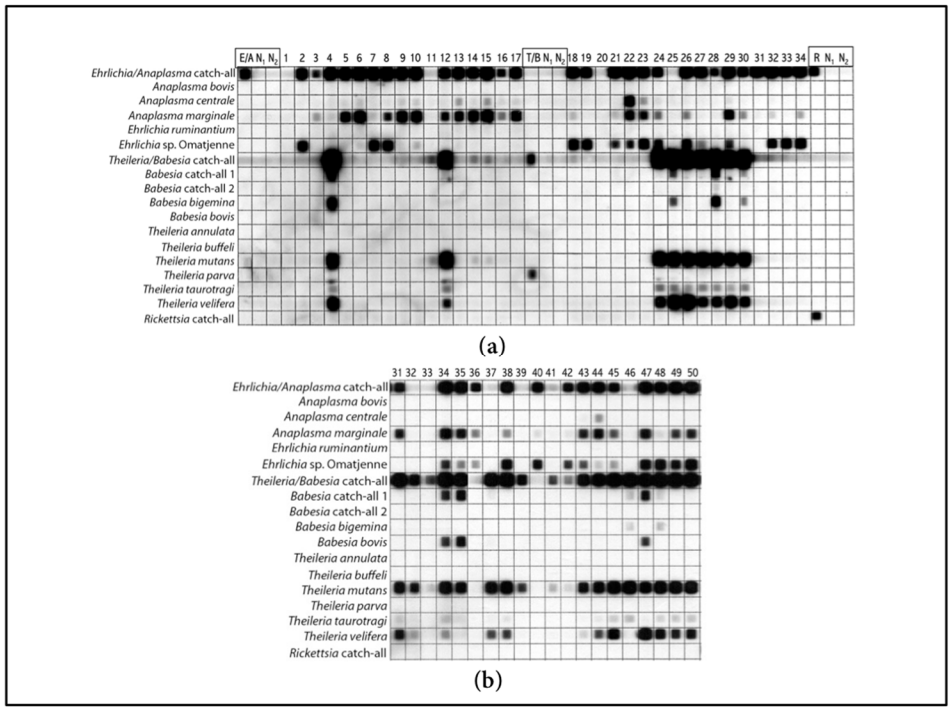

Figure 3.11 – Typical visualization of RLB results after x-ray development of hyperfilms, for villages of Badni **(a)**: samples 1–34, and Mangar **(b)**: samples 31–50.

The augmented signals seen in correspondence of the 'catch all' probes in samples infected by multiple species of the same genus (e.g. *Theileria*/*Babesia* catch-all in samples #4, 12 and 24–30 of Badni), can be a consequence of their increased concentration to the binding site compared to samples positive only for one single species. Sample #4 of Badni provides an example of 'doubtful' result, in this case with regards to *A. marginale* infection. Similarly, sample #6 of the same village is considered 'doubtful' for *A. centrale* infection, while sample #44 of Mangar is considered 'doubtful' for *T. taurotragi* positivity. As elucidated in paragraph 3.4.1, samples generating 'doubtful' results were re-processed by RLB.

[E/A = *Ehrlichia/Anaplasma* positive control (i.e. *Ehrlichia canis*); T/B= *Theileria/Babesia* positive control (i.e. *Theileria parva*); R = *Rickettsia* positive control (i.e. *Rickettsia africae*-like); N_1 = blank white paper negative control; N_2 = MilliQ water control].

3.4.2 Overall infection rates

561 animals out of 704 (82.5%, 95% CI: 79.2–85.9%), were found infected by at least one tick-borne haemoparasite. An overview of infection rates according to age classes (i.e. calves, juveniles, and adults) at the overall and village level is given by Table 3.6.

Table 3.6 – Proportion of infected animals within the sampled population.

Village Name	**Infected Animals/Animals sampled (%)**			
	Calves	**Juveniles**	**Adults**	**Totals**
Ampang West	0/0	28/33 (85%)	38/47 (81)	**66/80 (82.5)**
Badni	3/4 (75)	18/20 (90%)	46/56 (82)	**67/80 (84)**
Bokkos	6/6 (100)	22/25 (88)	42/49 (86)	**70/80 (87.5)**
Daffo	1/4 (25)	12/17 (71)	45/51 (88)	**58/72 (81)**
Hurti	1/2 (50)	7/9 (78)	64/69 (93)	**72/80 (90)**
Maiyanga	5/6 (83)	31/32 (97)	32/42 (32)	**68/80 (85)**
Mangar	3/5 (60)	25/29 (86)	44/46 (96)	**72/80 (90)**
Ruff	1/5 (20)	14/18 (78)	32/49 (65)	**47/72 (65)**
Tambes	6/11 (54)	0/1 (0)	35/68 (51)	**41/80 (51)**
Total	**26/43 (60)***	**158/184 (86)***	**378/477 (79.2)***	**561/704 (82.5)**

*Calves statistically less infected than juvenile (p=0.001) and adult cattle (p=0.023).

3.4.3 Comparison among study villages for infection rates

When comparing the nine study villages according to the number of infected and non-infected animals, only the village of Tambes resulted statistically significantly different compared to others ($p<0.001$) (Table 3.7).

Table 3.7 – Odds ratio, chi-square and p values originated from the comparison of villages according to their infection rates using the likelihood-ratio test, followed by Bonferroni correction.
For each village, the percentage of infected animals out of the sampled population (i.e. 'Infection rate') is also provided.

Village	Infection rate (%)	Odds ratio	Chi-square	P value
Ampang West	82.5	1.202	0.365	1.000
Badni	84	1.314	0.778	1.000
Bokkos	87.5	1.784	3.073	0.717
Daffo	81	1.056	0.031	1.000
Hurti	90	2.294	5.642	0.158
Maiyanga	85	1.444	1.357	1.000
Mangar	90	2.294	5.642	0.158
Ruff	65	0.479	7.214	0.065
Tambes	51	0.268	28.148	1×10^{-6}

3.4.4 Tick-borne haemoparasites prevalence

Theileria mutans was the most prevalent microorganism (n=435/704, 95% CI: 62.1–70.4%), followed by *Theileria velifera* (n=348/704, 95% CI: 47.9–56.9%), *Theileria taurotragi* (n=260/704, 95% CI: 35.1–43.9%), *Anaplasma marginale* (n=268/704, 95% CI: 34.8–43.5%), *Ehrlichia* species Omatjenne (n=239/704, 95% CI: 30.5–38.9%), *Babesia bigemina* (n=57/704, 95% CI: 5.6–10.2%), *Anaplasma centrale* (n=57/704, 95% CI: 4.2–8.3%), *Ehrlichia*/*Anaplasma* spp. (n=27/704, 95% CI: 2.1–5.7%), *Rickettsia* spp. (n=19/704, 95% CI: 1.7–5.2%), *Babesia bovis* (n=16/704, 95% CI: 1.0–2.9%), *Ehrlichia ruminantium* (n=8/704, 95% CI: 0.2–

1.9%; see Figure 3.12). None of the cattle sampled tested positive for *Anaplasma bovis*.

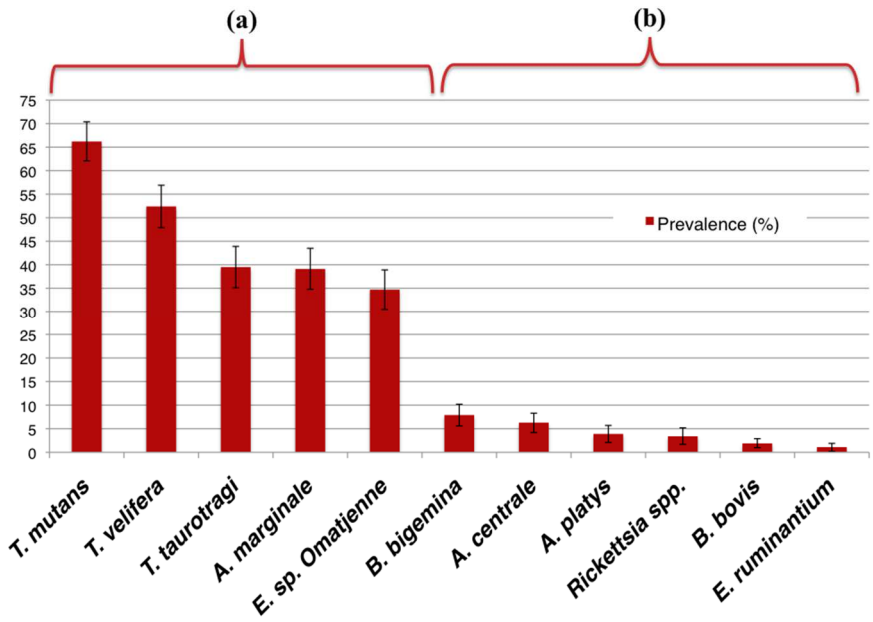

Figure 3.12 – Prevalence (%) of each tick-borne haemoparasite detected in the study area. Error bars on the histograms indicate 95% confidence intervals. Each tick-borne infection (TBI) represented underneath the curly bracket labeled as **(a)** was significantly more prevalent ($p<0.0001$) than each TBI featuring under curly bracket **(b)**.

3.4.5 Confirmation of amplicon identity

Sequenced *Ehrlichia*/*Anaplasma* catch-all positive samples were found 99-100% similar with *Anaplasma platys* (Accession No. KC989957.1, KF360842.1, KF576217.1) (n=9). Purified *Rickettsia* spp. 16S rRNA gene fragments (n=3) were found 100% similar with spotted fever group (SFG) rickettsiae (e.g. *Rickettsia massiliae*, Accession No. NR074486.1).

3.4.6 Single TBIs

Ninety-six single infections were detected, amongst 561 positive cases (12.3%, 95% CI: 10.3–15.6%), of which 9 were in calves (34.6% of positive animals), 27 in juveniles (17% of positive animals) and 60 in adult cattle (15.8% of positive animals). Cases of single infections were mostly represented by *T. mutans* (n=32), found in 7/9 villages, *E.* sp. Omatjenne (n=30) and *A. marginale* (n=22),

detected in all study villages, followed by *Rickettsia* spp. (n=6), *A. platys* (n=2), *B. bigemina* (n=2), *B. bovis* (n=1) and *T. velifera* (n=1) (Figure 3.13).

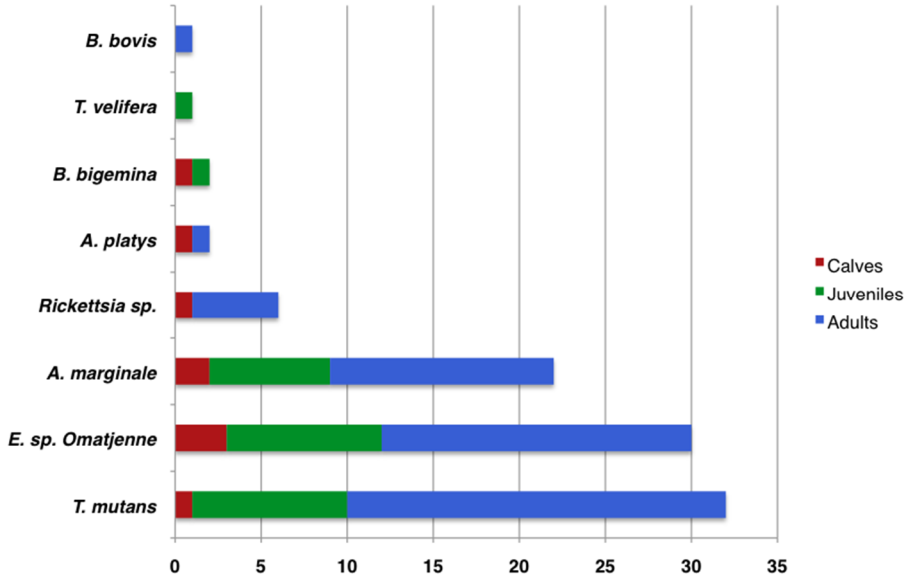

Figure 3.13 – Single TBIs according to age classes of tested animals (n=96).

3.4.7 Co-infection patterns

Overall 77 different species combinations were found. *Ehrlichia* (e.g. *E.* sp. Omatjenne) and *Anaplasma* species (e.g. *A. marginale*) were often seen associated with each other, as well as with the three *Theileria* species; also in presence of, although on a less frequent extent, *B. bigemina*. The latter species was never found infecting the same host together with *B. bovis*. In particular, the most frequent combinations including three (i.e. *T. mutans* + *T. taurotragi* + *T. velifera*) to five co-infective species (i.e. *A. marginale* + *E.* sp. Omatjenne + *T. mutans* + *T. taurotragi* + *T. velifera*).

Table 3.8 reports only the 12 most frequent co-infections. For a more detailed overview, the reader is referred to the extensive format of the table in the Appendix Section (Table 6.1). The largest variety of co-infections was recorded in adult cattle (n=58), followed by juveniles (n=43) and calves (n=11). In juveniles, most recurrent combinations consisted of the three *Theileria* species (i.e. *T. velifera* + *T. taurotragi* and *T. mutans*) alone and in association with *A. marginale* + *E. sp. Omatjenne* (Table

3.8). In adult cattle, as well as in juveniles, two of three most frequent co-infective species combinations were represented by the association of the three theilerias; and their combination with the association *A. marginale* + *E*. sp. Omatjenne; with the latter dual infection being as frequent as *T. mutans* + *T. velifera* (Table 3.8). Out of the 11 multiple combinations recorded, calves displayed a range of ten different co-infections, only one of which (i.e. *A. marginale* + *B. bigemina*) was recorded twice.

Table 3.8 – Twelve most frequent multiple infections by tick-borne haemoparasites according to age classes and overall number of animals.
(*Ac = Anaplasma centrale*; *Am = Anaplasma marginale*; *Ap = Anaplasma platys*; *E*spO = *Ehrlichia* sp. Omatjenne; *Er = Ehrlichia ruminantium*; *R = Rickettsia* spp.; *Bb = Babesia bigemina*; *Bbov = Babesia bovis*; *Tm = Theileria mutans*; *Tt = Theileria taurotragi*; *Tv = Theileria velifera*).

	Tick-borne haemoparasite species combinations	**Frequency**			**Totals**
		Calves	**Juveniles**	**Adults**	
1	*Tm_Tt_Tv*	1	13	65	79
2	*Am_E*spO_*Tm_Tt_Tv*	1	14	27	42
3	*Tm_Tv*	-	8	26	34
4	*Am_E*spO	-	4	29	33
5	*Am_Tm_Tt_Tv*	-	9	18	27
6	*Am_E*spO_*Tm_Tv*	-	6	15	21
7	*Ap_Tm_Tt_Tv*	-	2	10	12
8	*Am_E*spO_*Tm_Tt_Tv_Bb*	-	5	7	12
9	*Am_Tm_Tv*	-	2	9	11
10	*Am_E*spO_*Tm*	-	7	3	10
11	*Am_Ac_E*spO_*Tm_Tt_Tv*	-	1	9	10
12	*E*spO_*Tm_Tt_Tv*	-	-	10	10

Table 3.9 illustrates the infection patterns (i.e. single or multiple infections) found to be significantly ($p<0.001$) likely or unlikely to be detected in this study, following analysis through the association screening approach (Vaumourin *et al*., 2014). Ten infection patterns were found to be significantly likely to occur in this study, including 8 types of multiple infections; while 11 infection patterns were found to be significantly unlikely to be detected, including 9 multiple infection types (see Table 3.9).

Table 3.9 – Stastically significantly most and least likely infection patterns of the study. (*Ac = Anaplasma centrale*; *Am = Anaplasma marginale*; *Ap = Anaplasma platys*; *E*spO = *Ehrlichia* sp. Omatjenne; *Er = Ehrlichia ruminantium*; *R = Rickettsia* spp.; *Bb = Babesia bigemina*; *Tm = Theileria mutans*; *Tt = Theileria taurotragi*; *Tv = Theileria velifera*).

(A) Significantly most likely infection pattern (p<0.001)		
Infection pattern	**No. of observations**	**95% Confidence interval (%)**
Tm + *Tt* + *Tv*	79	10 – 44
Am + *E*spO + Tm + *Tt* + *Tv*	42	0 – 20
Am + *E*spO	33	1 – 22
*E*spO	30	3 – 28
Am + *E*spO + Tm + *Tt* + *Tv* + *Bb*	12	0 – 5
Ap + *Tm* + *Tt* + *Tv*	12	0 – 7
Am + *Ac* + *E*spO + Tm + *Tt* + *Tv*	10	0 – 5
Am + *Ac*	8	0 –7
Am + *Ac* + *E*spO	7	0 – 6
R	6	0 – 5
(B) Significantly least likely infection patterns (p<0.001)		
Am + *Tm*	7	11 – 46
Tm + *Tt*	6	8 – 44
*E*spO + *Tm*	5	6 – 38
Tv	1	10 – 47
Am + *Tm* + *Tt*	1	4 – 32
Am + *Tv*	0	4 – 30
*E*spO + *Tv*	0	3 – 33
Tt	0	3 – 31
Tt + *Tv*	0	2 – 30
Am + *Tt* + *Tv*	0	1 – 24
Am + *Tt*	0	1 – 23

3.4.8 Comparison of age classes

On the whole, calves were significantly less infected than juvenile (p=0.001) or adult cattle (p=0.023), whereas no statistically significant difference was detected between juveniles and adults (p=0.138) (data presented in Table 3.6). In particular, calves were significantly less infected than both juveniles and adults with regards to *T. mutans* (p<0.0001), *T. velifera* (p=0.001 and p<0.0001, respectively) and *T. taurotragi* (p<0.0001 and p<0.001, respectively), while no significant difference was recorded when comparing juvenile with adult cattle (p=0.3 for *T. mutans*, p=0.6 for *T. taurotragi*, and p=1 for *T. velifera*). In addition, calves were significantly less infected than juveniles (p=0.01), but not than adults (p=0.1) with *E.* sp. Omatjenne. Furthermore, both calves and juveniles were both significantly more infected by *B. bigemina* than adults (p<0.0001 and p=0.003, respectively). No *E. ruminantium* infection was detected in calves (see Figure 3.14).

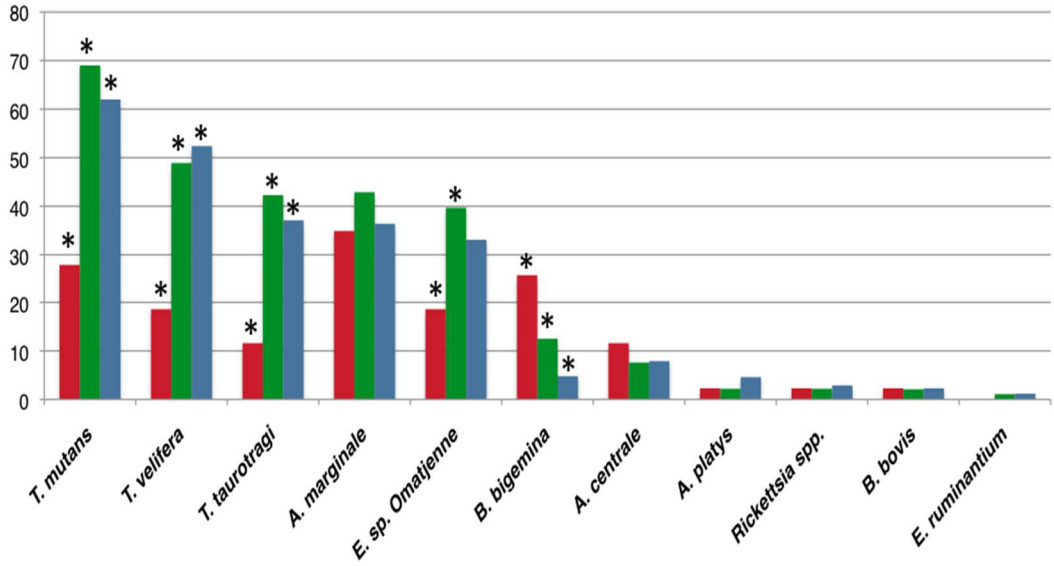

Figure 3.14 – Prevalence (%) of each tick-borne haemoparasite detected, compared according to age classes.

Red histograms = calves; green histograms = juveniles; blue histograms = adults). Asterisks indicate statistically significant difference between age classes.Calves were significantly less infected than both juveniles and adults for *T. mutans* (p<0.0001 in both cases), *T. velifera* (p=0.001 and p<0.0001) and *T. taurotragi* (p<0.0001 and p<0.001) and infections. With respect to *E.* sp. Omatjenne calves were significantly less infected than juveniles (p=0.01). For *B. bigemina* infection, both calves and juveniles were significantly more infected than adult cattle (p<0.0001 and p=0.003).

3.4.9 *A. marginale* sensitivity assessment

Results of the assessment of the analytical sensitivity of the RLB assay for the detection of *A. marginale* are shown in Figure 3.15, wherein a signal can be discerned up to at least dilution no. 11, equivalent to 4.3 *A. marginale* 16S copies/µl, for the dilution series prepared with milliQ water (Figure 3.15a), and up to dilution no. 10, equivalent to 43 copies/µl, for the series prepared with 1x TE adjusted with 30 ng/µl-concentrated bovine genomic DNA (Figure 3.15b).

As the 16S rRNA is a single copy gene in *A. marginale* and considering that each parasitized erythrocyte contains an average of 6 to 12 microorganisms (Eriks *et al*., 1989), the RLB here assessed was able to detect infection greater than seven *A. marginale*-infected erythrocyte/µl (taking into account the dilution series prepared using 1x TE adjusted with bovine genomic DNA) and/or greater than one *A. marginale*-infected erythrocyte/µl (taking into account the dilution series prepared using milliQ water). No cross-reactions with other *Anaplasma* or *Ehrlichia* species were observed.

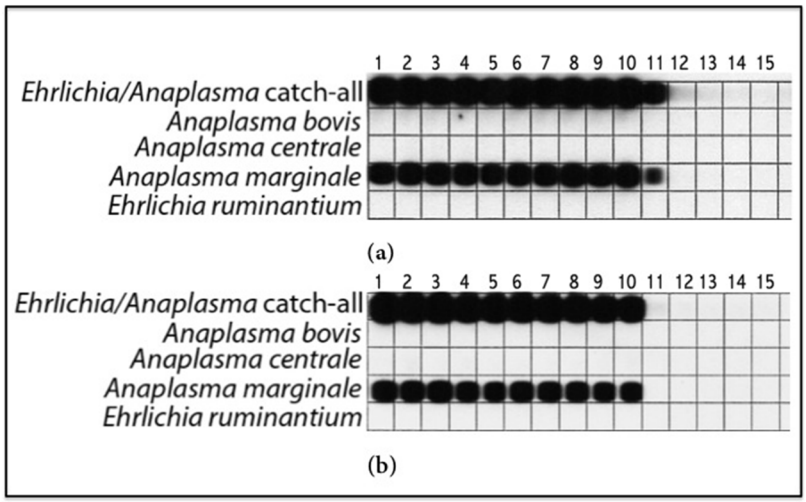

Figure 3.15 – Assessment of the analytical sensitivity of the RLB method for the detection of *Anaplasma marginale* using 15 ten-fold dilutions prepared in MilliQ water **(a)** and 1x TE buffer adjusted with bovine genomic DNA **(b)**. Initial DNA concentration (dilution #1) = 150 ng/µl, equal to 4.3×10^{10} *A. marginale* 16S rDNA fragment copies/µl.

3.5 Discussion

The present survey aimed to assess the occurrence of TBD-causing pathogens as well as less pathogenic tick-borne microorganisms in an area of Central Nigeria, where no acaricide-based vector control is usually undertaken in spite of the presence of a high tick species diversity and burden on cattle.

3.5.1 On the reverse line blotting (RLB)

In this study, an RLB-based method was employed to test each of 704 bovine whole blood samples against a panel of five genus- (i.e. 'catch-all') and 12 (excluding *T. parva*) species-specific probes. This represents the largest epidemiological survey for the detection of livestock TBIs carried out by means of RLB in the whole SSA, and also the first broad-scale molecular screening ever conducted in this field of research, in Nigeria.

The RLB is a 'semi-quantitative' diagnostic approach; when performed by sufficiently trained personnel, the intensity of the signals detected on the blot after hybridization, chemolumiscence and x-ray film development is affected by i) the concentration of template DNA and ii) that of the complementary probe covalently linked on the blotting membrane. In the case of this study, a working concentration of 400 µM was chosen for all probes used, so that the variability of intensity due to the latter factor could be minimized. Therefore, the variable intensity of signals seen on the spots formed on the RLB blots of this study is attributable to the varying concentration of DNA present in the samples. Not surpringly, in fact, the signals with the strongest intensity were seen in correspondence of the catch-all probes in samples infected by multiple species of the same genus (see Figure 3.11), seemingly due to the higher concentration of 'catch-all' DNA in the samples, shared by all microorganisms within the given genus.

In a few cases (n<10), the intensitity of the spots was very faint, so that the respective RLB outcome was considered as 'doubtful' (see also Figure 3.11). In these instances, a further RLB was carried out, using the same membrane after stripping it. If the same intensity of signals were obtained (i.e. very faint, but yet not negligible), samples were considered as 'positive'. Optimizing a kit relying on the

reading of RLB x-ray developments using digital imaging tools may help define a threshold of intensity of signals to be considered as 'cut-off' for positivity/negativity, making the interpretation of RLB results the most objective.

3.5.2 Epidemiological overview

This study disclosed high infection rates (>80%) in the overall cattle population, with a broad diversity of haemoparasite species detected, in presence of a complex scenario of multiple infections.

Comparing statistically the study villages amongst each other with regards to the number of infected and non-infected animals, the village of Tambes was found to differ significantly from the others ($p<0.001$). In this village, out of the 80 cattle sampled, 41 animals were found positive for any tick-borne microorganism, whilst 39 were found negative (overall infection rate=51%; see also Table 3.6).

The significantly lower number of infected animals in Tambes could possibly be attributed to its relative isolation from other villages where cattle are present (Majekodunmi, 2011). As a consequence, cattle in Tambes are therefore less likely to get in contact with herds from other villages and LGs, especially if compared to cattle from Bokkos LGA, where a number of short-distanced villages are present (i.e. Bokkos, Hurti, Daffo, Maiyanga and Mangar). The local cattle population of Tambes is reared according to year-round grazing in the surroundings of the village and on the rocky hills nearby (Majekodunmi[6], personal communication). Moreover, this village does not fall within any migration routes of cattle from other neighbouring villages (Majekodunmi[6], personal communication).

It is therefore thought that the lower opportunity of encountering cattle from other villages may have reduced the chances of exchange of ticks and the microorganisms they bear between herds, thus favouring the establishment of an epidemiological situation in which cattle are better able to halt the infections, bearing

[6] Dr Majekodunmi, Ayodele; 'Stamp Out *Sammore* (SOS)' programme coordinator, Welburn Research Group, Division of Pathway Medicine, School of Biomedical Sciences, the University of Edinburgh, Edinburgh, UK.

either no parasitaemia or parasitaemia lower than the detectability threshold via RLB.

In fact, in villages from Bokkos LGA, the overall infection rates ranged from 81% (n=58/72), in Daffo, to 90% (n=72/80), in Hurti and Mangar, including the 85% (n=68/80) of Maiyanga and 87.5% (n=70/80) of Bokkos (see Table 3.6). As opposed to Tambes (located in the LGA of Pankshin), the LGA of Bokkos falls within the migration route of cattle originating from other neighbouring LGAs of the Plateau State (usually the northern part, and moving in south-west-bound direction) Majekodunmi, 2011), possibly contributing to the reassortment of ticks and TBIs in this part of the study area.

Inevitably, the significantly lower infection rates affected the prevalence detected for each individual tick-borne infection detected in the village of Tambes (see Table 3.10). Nonetheless, though their prevalence was nearly halved compared to the overall trend, also in this village, the most frequently detected microorganisms included *T. mutans* followed by *T. velifera, T. taurotragi* and *A. marginale* (see Table 3.10).

Table 3.10 – Prevalence of each tick-borne migroorganism detected in this study, in the overall study area and in the village Tambes.

	Prevalence (%) of tick-borne infections										
	T. mutans	***T. velifera***	***T. taurotragi***	***A. marginale***	***E.* sp. Omatjenne**	***B. bigemina***	***A. centrale***	***A. platys***	***R. massiliae***	***B. bovis***	***E. ruminantium***
Overall study area	66.2	52.4	39.5	39.1	34.7	7.9	6.3	3.9	3.5	2.0	1.1
Tambes	34.0	22.5	18.8	15.0	11.2	2.5	-	5.0	2.5	3.8	-

Though not statistically significant (p=0.065), a considerably lower number of cattle were found infected (n=47/72, 65%) in the village of Ruff. This could probably be due to the low total cattle population in this village (n=154, see also Table 3.1), possibly contributing to a lower tick abundance on the pastures, being that dependent on host density (Sonenshine, 1993). Moreover, hosting low number of

cattle in a village may also turn advantageous from a nutrition standpoint, with less competition among cattle to access suitable pastures and, when available, crop residues, an aspect particularly critical on the Plateau during the dry season (Majekodunmi, 2011).

Looking at all tick-borne haemoparasite species detected, this study established the existence of a stark dichotomy in their occurrence, with a group of five microorganisms (i.e. *T. mutans*, *T. velifera*, *T. taurotragi*, *A. marginale*, *E.* sp. Omatjenne) being significantly ($p<0.0001$) more prevalent (>30%), than *A. centrale*, *B. bigemina*, *A. platys*, *Rickettsia* spp., *B. bovis*, and *E. ruminantium*, being below 10% in prevalence. In Table 3.11, each haemoparasite detected in this study is put in relation to its tick vectors, either ascertained or yet to be confirmed for Nigeria. Further tick-pathogen associations, in relation to the TBDs-causing microorganisms found in Nigeria have been previously presented in Table 1.2.

Table 3.11 – Prevalence (%) of tick-borne haemoparasites detected and their main tick vectors.

Tick-borne microorganism		**Prevalence (95% CI)**	**Likely or ascertained tick vector in the study area**	**Reference (if available)**
Significantly more prevalent ($p<0.0001$)	*Theileria mutans*	66.2% (62.1–70.4)	- *Amblyomma variegatum*	Uilenberg *et al.* (1974); Young *et al.* (1978)
	Theileria velifera	52.4% (47.9–56.9)		
	Theileria taurotragi	39.5% (35.1–43.9)	- *Rhipicephalus* spp.†	N/A
	Anaplasma marginale	39.1% (34.8–43.5)	- *Rhipicephalus* (*Boophilus*) spp. - *Rhipicephalus simus* Group†	Samish *et al.* (1993); Gueye *et al.* (1994)
	Ehrlichia sp. Omatjenne	34.7% (30.5–38.9)	- *Hyalomma truncatum*†	Du Plessis (1990)
Significantly less prevalent ($p<0.0001$)	*Babesia bigemina*	7.9% (5.6–10.2)	- *Rhipicephalus* (*Boophilus*) spp.	Adetunji *et al.* (1981); Akinboade *et al.* (1981)

Tick-borne microorganism		Prevalence (95% CI)	Likely or ascertained tick vector in the study area	Reference (if available)
	Anaplasma centrale	6.3% (4.2–8.3)	- *Rhipicephalus* (*Boophilus*) spp. †; - *Rhipicephalus simus* Group†	Shkap *et al*. (2009)
	Anaplasma platys	3.9% (2.1–5.7)	- *Rhipicephalus sanguineus* Group†	Simpson *et al*. (1991); Ramos *et al*. (2014)
	Rickettsia massiliae	3.5% (1.7–5.2)		Beati and Raoult (1993)
	Babesia bovis	2.0% (1.0–2.9)	- *Rhipicephalus* (*Boophilus*) spp. †;	Akinboade and Dipeolu, (1981,1983); Büscher (1988)
	Ehrlichia ruminantium	1.1% (0.2–1.9)	- *Amblyomma variegatum*	Walker and Olwage (1987); Bezuidenhout, (1987)

†Yet to be ascertained for the study area.

As reviewed in Table 3.11, some of the most (e.g. *Theileria mutans* and *Theileria velifera*) and least prevalent (e.g. *E. ruminantium*) microorganisms (and pathogens) of this study share the same tick vectors (e.g. *Amblyomma variegatum*).

Therefore, in the light of the abundance of the competent vectors of most of these microorganisms in the study area at the time of the year when the blood sampling took place (Lorusso *et al*., 2013a; see also Chapter 2), one must take into account factors that lie outside the mere a) 'arthropod sphere', encompassing necessarily b) the vertebrate hosts and its immunity (i.e. colostral, innate, active, genetic); c) possible competition amongst organisms, especially within the same genus; d) intrinsic properties of the diagnostic methods employed (e.g. sensitivity and specificity).

The high prevalence recorded for *T. mutans*, *T. velifera*, *T. taurotragi*, *A. marginale* and *E.* sp. Omatjenne undoubtedly suggests the existence of a constant

challenge to indigenous cattle in the Nigerian Plateau by their tick vectors. This is likely to lead, in the long term, to the development of a rather steady immunity in animals recovering from active infections (reviewed by Jonsson *et al*., 2012). This is supported by the fact that most of the animals surveyed in this study (i.e. cattle older than two years) did not present any clinical signs referable to any of the TBDs caused by the most common microorganisms (i.e. anaplasmosis and/or theileriosis), thus suggesting a scenario of 'endemic stability', with high infection rates and antibody titres, with low incidence of clinical disease in the surveyed population (Coleman *et al.*, 2001).

The possible presence of 'endemic stability', however, should not be hypothesized only for those tick-borne infections recorded in high prevalence and lack of clinical signs. These are indeed tick-borne infections characterised by the establishment of a rather steady post-recovery 'carrier' state in immune hosts (e.g. theileriosis, anaplasmosis) that will continue living in apparently healthy conditions (Jonsson *et al*., 2012). However, in other cases, such as babesiosis, recovery from the acute infections is followed by a latent infection characterised by a recrudescent parasitaemia occurring at irregular intervals (reviewed by Bock *et al*., 2004) reaching values as low as 10^{-8}% (Calder *et al*., 1996). Moreover, it should also be noted that carrier animals have already seroconverted and their circulating antibodies may evoke antigenic variations of the parasites (Allred, 1995; 2001). This may have affected the results here recorded for *Babesia* species, considering that the RLB sensitivity was ascertained to be of 10^{-6}% for *B. bovis* (Gubbels *et al*., 1999).

A more detailed discussion on each haemoparasite detected will be addressed in the following sections.

3.5.3 Infection rates vs tick burdens

In Chapter 2, 9 villages were compared with each other on the basis of the mean tick burdens recorded on their cattle (see also Section 2.4.2). In Chapter 3, the same 9 villages were compared with each other with regards to the infection rates of their cattle (i.e. positivity vs negativity for any tick-borne microorganism detectable via the RLB assay implemented) (see also Section 3.4.3)

Though the sampling times of the studies presented in Chapter 2 and 3 were different (i.e. September-October 2010 for Chapter 2 and October 2008 for Chapter 3) some indications could be drawn, based on the fact that species acting as vectors of the major bovine tick-borne pathogens (i.e. *Anaplasma* spp., *Babesia* spp. and *Theileria* spp.) were found in all 9 study villages.

In Chapter 2, cattle from the villages of Ampang West and Maiyanga were significantly less infested by ticks than those from a few other villages (i.e. Badni, Daffo and Mangar for both villages, Hurti for Ampang West only), when considering the overall mean tick burdens of infestation. Conversely, cattle from the village of Mangar were significantly more infested than those from a few other villages (i.e. Ampang West, Bokkos, Hurti, Maiyanga, Ruff and Tambes). Looking at results from Chapter 3, the villages of Ampang West and Maiyanga were characterized by rather high infection rates (n=66/80, 82.5% and n=68/80, 85%, respectively). Moreover, 10 of the 11 tick-borne microorganisms detected in the study area were found in Ampang West (no *B. bovis*) and 9/11 (no *E. ruminantium* and *B. bovis*) in Maiyanga were found in either of the villages). Moreover, Ampang West was the village where the highest prevalence of *E. ruminantium* was recorded in the study area (i.e. 6.9% vs 1.1% of the entire study area).

This suggests that the exposure of animals to several TBIs in this area may not necessarily be related to high tick burdens.

Infection rates in Mangar were high (n=72/80, 90%), especially in adult cattle (n=44/46, 96%), found considerably infested by ticks (i.e. 53.8 ± 10.9), especially if compared to the overall mean (i.e. 22 ± 1.4) and the adults' mean tick burden (i.e. 23.6 ± 1.6). In this village 10 of the 11 tick-borne haemoparasites were found infecting cattle (no *E. ruminantium* in this case).

Looking at the infections rates documented in Chapter 3, the village of Tambes was found significantly less infected than others ($p=1\times10^{-6}$), and the village of Ruff, was also found characterized by a lower, though not significant statistically (p= 0.065), infection rate. Looking at the tick burdens documented in Chapter 2, both villages were characterized by mean tick loads (18.8 ± 4.4 for Tambes and 21.5 ± 3.65 for Ruff) very close to the overall mean of the entire study area (i.e. 22 ± 1.4).

This finding, together with the fact that 9 of the 11 TBIs detected in the study area were found in these two villages (no *A. centrale* and *E. ruminantium* in Tambes; no *E. ruminantium* and *B. bovis* in Ruff), further support what hypothesized for Ampang West and Maiyanga.

3.5.4 *Theileria mutans* and *Theileria velifera* – two 'long known' theilerias in Nigeria

Theileria mutans and *T. velifera* were the two most prevalent microorganisms (66.2% and 52.4%, respectively). These two non-pathogenic *Theileria* species have long been recognised to be the only two *Theileria* species present in Nigeria (Perié *et al*., 1979; Saidu *et al*., 1984). Both *T. mutans* and *T. velifera* are transmitted by *Amblyomma variegatum* (Uilenberg *et al*., 1974; Young *et al*., 1978), endemically present in the whole of Nigeria (Maina and Bayer, 1984) including the Plateau State (see Chapter 2 and Lorusso *et al*., 2013a).

The overall very high prevalence recorded in this survey for both *T. mutans* and *T. velifera* agreed with previous RLB-based studies carried out in other SSA countries such as Uganda (Oura *et al*., 2004) and South Sudan **(**Salih *et al*., 2007**)**. Previous studies carried out in mid 1970s and '80s in Nigeria recorded a *T. mutans* prevalence, at microscopical examination of blood smears, ranging from 32 to 76% according to the period of the year (Dipeolu, 1975b; Saidu, 1981; Saidu *et al*., 1984).

Although in different proportions, these two *Theileria* species were recorded in all age classes, being the second (i.e. *T. mutans*) and fourth most prevalent (*T. velifera*) haemoparasites detected in calves, and first and second most prevalent in both juveniles and adults. This suggests an early exposure of cattle on the Plateau with these piroplasms, due to early infestations with *A. variegatum*. Nevertheless, although presenting some positive cases, calves presented significantly less infections by both *Theileria* species than juveniles and adults (Figure 3.14), a finding that is also in agreement with previous other studies. Recently in Uganda, the age of nine months was identified as the 'cut-off' period in cattle, this was determined at the point at which most animals within the same herd are infected by *T. mutans* and *T. velifera* (as well as *T. parva* and *A. bovis*) (Asiimwe *et al*., 2013). In this PhD survey,

animals identified as 'calves', aged six months or less, disclosed a prevalence of infections of 27.9% (*T. mutans*) and 18.8% (*T. velifera*) (Figure 3.14). Interestingly, the same work in Uganda (Asiimwe *et al*., 2013) as well a cytology-based survey in Ghana (Bell-Sakyi *et al*., 2004) ascertained the persistence of positivity in cattle after first infection, being fully in line with the high prevalences recorded in the present study for both *T. mutans* and *T. velifera* in juvenile (69.0 and 48.9%, respectively) and adult cattle (62% and 52.4%).

3.5.5 *Theileria taurotragi* – a 'novel' *Theileria* species in West Africa?

The present work represents the first evidence of the presence of *T. taurotragi* in West Africa. Primarily associated with elands (*Taurotragus* (*Tragelaphus*) *oryx* (Pallas, 1766)) in East (Oura *et al*., 2011) and Southern Africa (Uilenberg *et al*., 1982), *T. taurotragi* can still infect domestic ruminants, such as cattle and sheep (Uilenberg *et al*., 1982) and to date it has been recorded in cattle from East, central, and southern SSA (Norval *et al*., 1992). This *Theileria* species is known to be transmitted naturally by *Rhipicephalus appendiculatus* (Young *et al*., 1980) and *Rhipicephalus zambesiensis* (Lawrence *et al*., 1983), and, experimentally, by *Rhipicephalus pulchellus* and *Rhipicephalus evertsi* (Young *et al*., 1977b; Lawrence *et al*., 1983; Norval *et al*., 1992). Not only is its original wildlife host not present in West Africa, but also none of the aforementioned tick species can be found in this part of Nigeria (see Chapter 2), with *Rh. evertsi evertsi* being retrieved only more southward (Reye *et al*., 2012). Although fatal infections had been recorded in elands (Brocklesby, 1962; Grootenhuis *et al*., 1980), *T. taurotragi* is usually associated with mild to sub-clinical conditions in cattle, with neurological syndromes (so-called 'turning sickness') being seldom documented (Lawrence and Williamson, 2004**)**. Therefore, an exchange of 'competent' multiple–host ticks from an infected antelope similar to *T. oryx* to cattle is the hypothesis here raised to explain the presence of *T. taurotragi* in cattle in Nigeria.

Even though the common eland is not present in the whole of West Africa, several other antelope species may be found roaming freely across the forested areas

of the Plateau State, including oribis (*Ourebia ourebi*), bushbucks (*Tragelaphus scriptus*), red-flanked duikers (*Cephalophus rufilatus*), grey duikers (*Sylvicapra grimmia*), klipspringer (*Oreotragus oreotragus porteousi*) and, to a less frequent extent, Buffon's kobs (*Kobus kob kob*) and korrigums (*Damaliscus lunatus korrigum*) (Happold, 1971; Anadu, 1987). Other antelope species (i.e. Bohor reedback, *Redunca redunca*; waterbuck, *Kobus ellipsiprymnus defassa*; roan antelope, *Hippotragus equinus*; western hartebeest, *Alcelaphus buselaphus major*) are also native of the Nigerian northern Guinea vegetation zone, but their presence in the wild has been severely endangered over the past three decades by uncontrolled hunting, agricultural expansion and livestock grazing (East, 1990). Therefore, their current distribution is limited to the conservation areas of this vegetation zone (e.g. Wase Game Reserve; Yankari Game Reserve; Pai River Game Reserve; Pandam Wildlife Park) (East, 1990) where the Fulani cattle herds are unlikely to graze.

Moreover, an antelope species close to *T. oryx*, the so-called Eastern Giant Eland *Tragelaphus* (*Taurotragus*) *derbianus* (Gray, 1847), was originally present in several West (i.e. Guinea, Mali, Senegal), Central (i.e. Cameroon, Central African Republic, Congo, Democratic Republic of the Congo) and East African countries (i.e. Chad, Sudan, Uganda). This animal may occasionally be found within the Nigerian territory in proximity of the border with Cameroon (East, 1990; IUCN, 2013). In West Africa, the giant eland usually prefers woodlands and forested Guinean savannas, localizing in hilly and rocky areas in the vicinity of water sources (IUCN, 2013). Although these are habitats of which the Plateau State is plentiful, it is not likely that this species' roaming would reach up to the northern Guinea zone of Central Nigeria. Although, none of the cattle herds screened in this study seemingly reached eastern Nigeria (i.e. neighbouring the Cameroonian border) over a migration period, it cannot be excluded that, historically, the Fulani cattle could have shared pastures with *T. derbianus* in this part of the country. This could have favored the exchange of infected ticks from antelopes to cattle.

Among the ticks recorded in the survey of Chapter 2, it would be advisable to assess the occurrence of *T. taurotragi* in the three-host rhipicephalinae such as the highly prevalent *Rhipicephalus guilhoni* and *Rhipicephalus simus* Group, as well as

the more infrequent *Rhipicephalus lunulatus*, *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* (Lorusso *et al*., 2013a).

3.5.6 *Anaplasma marginale*

Anaplasma marginale was the fourth most prevalent haemoparasite detected in this study. Inevitably, the prevalence here recorded (39.1%) was considerably higher than those from previous studies carried out in Nigeria in the '70s (i.e. 9.9–17.7%) (Dipeolu, 1975b; Leeflang and Ilemobade, 1977a) as well as in more recent times (i.e. 1.9%) (Kamani *et al*., 2010**)**, based on direct cytological examination of Giemsa-stained blood smears. The present prevalence is, however, similar to that of 34% found in the late '70s by the more sensitive serological rapid card agglutination test in a similarly sized cattle population (n=573) from Northern Nigeria (Obi, 1978). Similarly, another serological investigation carried out later on in 50 herds from ten states in Northern Nigeria had disclosed a prevalence of 79.4% of *A. marginale*, (Ajayi and Dipeolu, 1986). Such results are still consistent with the present findings, considering that the use of the RLB can enable either detecting an active (and/or recurrent, in the case of rickettsiae) infection or a carrier status, but not recovered cases were seroconversion has occurred and the infection has been cleared, detectable only by means of serology.

The overall prevalence recorded in this study for *A. marginale* was rather uniformly distributed among age classes (see Figure 3.14), ranging from 34.9% in calves to 42.9% in juveniles and 36.4% in adults. Interestingly, in this study *A. marginale* was the most prevalent microorganism amongst calves, being detected in 15 of the 26 positive cases, possibly reflecting the principle according to which when the inoculation rate of haemoparasites is adequate to ensure that all young animals are infected while they are protected by colostral and/or immunity, clinical disease is minimal and endemic stability is achieved (Norval *et al*., 1992). It is therefore likely that these young calves represent the only cases, amongst those positive, of active infections, while other older cattle could be considered as carriers. Recovery from acute anaplasmosis results indeed in a persistent infection status characterised by repetitive cycles of rickettsiaemia (Eriks *et al*., 1993). Importantly, in the case of *A.*

marginale, persistently infected cattle serve as long-term reservoirs to disseminate the transmission within herds (Eriks *et al*., 1993), not only via the competent ticks *Rhipicephalus* (*Boophilus*) *annulatus* and *Rhipicephalus* (*Boophilus*) *decoloratus*), highly prevalent in the area of this study (Chapter 2 and Lorusso *et al*., 2013a), but also via other mechanical vectors, such as blood-sucking flies (*Tabanus* spp., *Stomoxys* spp.) (Foil, 1989; Scoles *et al*., 2005, Baldacchino *et al*., 2013), also found in this part of Nigeria (Glover, 1967; Olaniyan[7], personal communication) as well as via iatrogenic transmission (Reeves and Swift, 1977).

This is inevitably of epidemiological relevance as it poses a high risk for a potential outbreak of clinical anaplasmosis in the event of the introduction of exotic taurine breeds to this region. Furthermore, Fulani pastoralists from the area habitually report cases of abortions (Majekodunmi[8], personal communication) possibly attributable to *A. marginale* infection (Fowler and Swift, 1975) in presence of other triggering factors (e.g. malnutrition, trypanosomiasis, other concomitant TBIs etc.).

3.5.7 *Ehrlichia* species Omatjenne – a highly prevalent, poorly known tick-borne haemoparasite

This study represents the first record of *E.* sp. Omatjenne in cattle in Nigeria. This poorly known *Ehrlichia* species was initially isolated in *Hyalomma truncatum* ticks in Southern Africa (Du Plessis, 1990). The acarological survey elucidated in Chapter 2 of this thesis confirmed indeed the presence of this tick species in the study area. Initially thought to be apathogenic in cattle, studies have instead showed its association, in experimental conditions, with 'heartwater'-like syndromes (Du Plessis, 1990; Allsopp *et al*., 1997). If the involvement of *E.* sp. Omatjenne in

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cowdriosis was confirmed, the low prevalence detected here for *E. ruminantium* (1.1%) would be more plausible in the light of the much higher prevalence (34.7%) detected for this poorly known *Ehrlichia* species. In this respect, the finding in the present study of juveniles being significantly more infected (with a prevalence nearly twice as high) than calves (see Figure 3.14) suggests that cattle are more likely challenged by the infection between 6 months and 2 years, as a possible result of the declining of a previous colostral and perhaps also innate immunity, as seen for *E. ruminantium* infections.

Moreover, the prevalence in the adult cattle only slightly lower than that of juveniles (33.1% and 39.7%, respectively), suggests the persistence of a carrier status in older animals, as already proven for *E. ruminantium* (Andrew and Norval, 1989). However, considering the remarkably higher prevalence recorded for *E.* sp. Omatjenne, a more 'steady' rickettsiaemia should expected to be occurring in post-active infection carriers of this microorganism.

Future studies aiming to assess clinical relevance of *E.* sp. Omatjenne in cattle as well as in small ruminants in the Plateau would be advisable.

3.5.8 *Anaplasma centrale*

This study also provides the confirmation of the presence of *A. centrale* in Central-Northern Nigeria, with a prevalence of 6.3%. Considering the richness of competent vectors (i.e. *Rh.* (*Bo.*) *annulatus*, *Rh.* (*Bo.*) *decoloratus*, *Rh. simus* Group) of *A. centrale* species in the study area, as shown in Chapter 2 (Lorusso *et al*., 2013a), one may speculate on the lower likelihood of this species to disseminate within herds, compared to *A. marginale*.

Moreover, as an endemically stable setting for *Anaplasma* spp. infections is usually characterised by high infection rates in adult cattle, due to their carrier status (see the case of *A. marginale* infection in this survey), the rather homogenous prevalence detected for *A. centrale* in this study across all age classes (calves: 11.6%; juveniles: 7.6%; adults: 7.9%, see Figure 3.14) suggests an extent of epidemiological 'instability' for the latter species. This situation may favor the onset of sporadic episodes of acute anaplasmosis in the indigenous cattle population.

3.5.9 *Babesia bigemina*

As early as in the 1920s, bovine babesiosis was known to be endemic in most of Nigeria, with cattle becoming infected in early life without showing apparent infection, then acquiring life-long immunity after recovery, due to repeated challenge by *Rh.* (*Bo.*) *decoloratus* ticks (Leeflang and Ilemobade, 1977a); the most prevalent tick species in the survey described in Chapter 2 (Lorusso *et al*., 2013a). This would explain the overall low prevalence (7.9%) recorded in this study for *B. bigemina*, undoubtedly affected by the lower infection rates recorded in the more numerous adults. Here, in fact, calves (25.6%) and juveniles (12.5%) were found significantly more infected than adult cattle (4.8%) ($p<0.0001$ and $p=0.003$, respectively).

In the case of *Babesia* infection, passive colostral immunity usually lasts for approximately two months, being followed by the onset of innate immunity at a time ranging between three to nine months of age (reviewed in Jonsson *et al*., 2012). This non-specific innate immunity usually involves activated-monocytes, macrophages, natural killer cells and neutrophil-released factors such as interleukin (IL)-12, IL-1β, interferon (IFN)-γ, tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) (Shoda *et al*., 2000; Goff *et al*., 2001). Moreover, work carried out in a South African ranch with non-intensive tick control, identified the age of seven-eight months in calves as the period when seroconversion against *B. bigemina* starts (Regassa *et al*., 2003). Therefore, calves exposed to *Babesia* infection during the first six to nine months of their life rarely show clinical sign of babesiosis, developing thereafter a solid long-lasting humoral immunity.

Therefore, the higher levels of infection observed in calves and juvenile cattle compared to adults in this study ($p<0.0001$ and $p=0.003$, respectively) can be seemingly considered as individuals in which passive and innate immunity has declined, being exposed to sufficiently high challenge by infective *Rh.* (*Bo.*) *decoloratus* ticks and thus developing detectable parasitaemia.

Importantly, as opposed to the case of *A. marginale*, *T. mutans* and *T. velifera*, previous worked showed that latent *B. bigemina* infections, following the first active exposure, seldom persist for longer than a year, with cattle remaining infective for ticks only for two months (Lay *et al*., 1978; Zintl *et al*., 2005).

It is therefore likely that juvenile and adult cattle testing negative in this study included large proportions of animals that had successfully recovered from active infections and had developed protective humoral immunity. In a study carried out in the South Sudan, aiming to compare the RLB with an enzyme-liked immunosorbent assay (ELISA), 46 out of 90 samples tested positive for *B. bigemina*, while no DNA was detected at the RLB (Salih *et al*., 2007).

Therefore, if juvenile and adult cattle of the present survey had been tested serologically, it is possible they would have displayed high seroconversion rates.

3.5.10 *Babesia bovis* and its still uncertain vector in Nigeria

The present study provides the molecular confirmation of the occurrence of *B. bovis* in Central-Northern Nigeria. Several other studies had reported the presence of *B. bovis* in cattle in this part of the country relying on morphological (Leeflang and Ilemobade, 1977a,b; Kamani *et al*., 2010) and serological characterization (Akinboade and Dipeolu, 1984).

In this study, *B. bovis* was found at a lower prevalence (1.9%) than that of *B. bigemina* (7.9%), especially in calves (n=1 and n=11, respectively). This could be explained considering that tick infection rates are usually higher with *B. bigemina* (0.23%) than in *B. bovis* (0.04%), (Mahoney and Mirre, 1971), with a consequent less frequent rate of transmission of the latter to cattle. This would also suggest that, in an area where both species are present, endemic stability would more likely be establish for *B. bigemina* (Bock *et al*., 2004). Moreover, with special regards to the adult cattle population, the lower prevalence of *B. bovis* (2.3%) compared to *B. bigemina* (4.8%) could be also explained by the seemingly lower parasitaemia levels occurring in carrier animals in case of the former species (Gubbels *et al*., 1999).

In this study, no case of co-existence of the two *Babesia* spp. was recorded in any of the cattle examined (n=704), in accordance with another RLB-based study in 477 cattle in Mozambique (Martins *et al*., 2010). Besides the aforementioned discrepancy in transmission rates between *B. bigemina* and *B. bovis* by *Boophilus* ticks, another possible justification for the lack of co-infections detected could be the

hypothetical existence of competition between *B. bigemina* and *B. bovis*. However, while *Rh.* (*Bo.*) *decoloratus* is known as vector for *B. bigemina* in Nigeria (Adetunji *et al*., 1981), the vector capacity for *B. bovis* in this country has not yet been fully clarified. A tick species that could plausibly be involved in the transmission of *B. bovis* in Nigeria is *Rhipicephalus* (*Boophilus*) *annulatus* known for being a vector of this piroplasm in other geographic areas (e.g. Southern Europe and Northern Africa) (Bock *et al*., 2004), and well represented in the Plateau State (see Chapter 2 and Lorusso *et al*., 2013a). However, a study carried out in the south of the country over 30 years ago proved that another boophilid, *Rhipicephalus* (*Boophilus*) *geigyi* harboured kinetes associated for shape and size with *B. bovis*, in both eggs and larvae that eventually infected splenectomised calves (Akinboade and Dipeolu, 1981). If it was confirmed also for the central-northern part of the country, knowledge on this tick-pathogen association would help explain the lower prevalence of *B. bovis* compared to *B. bigemina*, considering that *Rh.* (*Bo.*) *geigyi* is not as prevalent (7.8%) as *Rh.* (*Bo.*) *decoloratus* (41.4%) and *Rh.* (*Bo.*) *annulatus* (15.4%) in the Nigerian Plateau State (Chapter 2).

The current epidemiological scenario, certainly requiring additional investigations, may be further complicated, as already discussed previously, by the introduction in Nigeria of the invasive 'pantropical cattle tick' *Rhipicephalus* (*Boophilus*) *microplus*. Therefore, considering the homogenously low occurrence of *B. bovis* across all three age classes (calves: 2.3%, juveniles: 2.1%, adults: 2.1%; overall: 1.9%, see also Figure 3.14), as well as the complex epidemiological features here discussed, the involvement of *B. bovis* in the sporadic cases of 'redwater fever' reported by pastoralists and veterinary personnel visiting the study villages throughout the wet season, cannot be ruled out.

3.5.11 *Ehrlichia ruminantium* – understanding such a low prevalence

The very low prevalence (i.e. 1.1%) detected for *E. ruminantium* can be attributed to several causes. This microorganism is the causative agent of heartwater in both cattle and small ruminants in SSA and the Caribbean, as well as several

islands of the Indian Ocean including Madagascar (Provost and Bezuidenhout, 1987), and it is known to be widespread in Nigeria (Leeflang and Ilemobade, 1977a,b).

In West Africa, heartwater cases follow a dual mode per year, and are usually expected during the periods of nymphal (i.e. dry season) and adult activity (i.e. onset of the rains) of its competent vector *A. variegatum*, in which transovarial transmission does not occur (Sumption, 1996). In Central Nigeria, the abundance of adult stages of this tick species peaks in the month of June (i.e. early rains), decreasing then until September and increasing again, although to a lower extent, from October to November (i.e. late wet season) (Bayer and Maina, 1984).

Interestingly, all 8/704 animals found positive in this study were either juvenile (n=2) or adult cattle (n=6). This can be explained by taking into account endemic stability, that also for cowdriosis, is attributable to a combination of factors such as: vertical transmission (Deem *et al*., 1996a), colostral (Deem *et al*., 1996b) and innate immunity in calves, presence of carrier animals and transmission routes within the tick vector (Deem *et al*., 1996a; see Section 1.2.3).

Considering that none of the eight *E. ruminantium*-positive cases of this survey displayed any clinical sign referable to heartwater, it is likely these animals were carriers of this rickettsia that had recovered from previously active infections. During acute infection, in fact, taking place by the time the innate immunity in the young animals decreases, the number of circulating rickettsiae is usually high during the febrile reaction and for a period thereafter (e.g. up to 40 days) (Ilemobade, 1978; Andrew and Norval, 1989), though some variability of the bacteraemia was seen according to the strain of the pathogen (Jongejan *et al*., 1989).

During the rickettsiaemia, *E. ruminantium* is found in the circulating neotrophils (in the stage of 'morula'; Logan *et al*., 1987). The parasitized neutrophil usually disrupts five to six days after the beginning of its infection, liberating several *E. ruminantium* 'elementary bodies' that will parasitize then other circulating neutrophils and the endothelial cells (Jongenjan *et al*., 1991).

After recovery from the acute phase, low numbers of microorganisms can still reproduce in the endothelial cells of the capillaries, being released only periodically into the bloodstream (Andrew and Norval, 1989). This would explain

why not all carrier animals can be diagnosed as positive from screening of blood samples, and also why ticks are able to pick up infections from individual animals only sporadically (Andrew and Norval, 1989).

Another further reason for such a low prevalence could be represented by a limitation of the RLB. A study on the molecular detection of *E. ruminantium* in *A. variegatum* ticks from The Gambia compared a nested 16S-based RLB (employing the same *E. ruminantium*-specific probe employed in this survey, but a different primer set) with two other nested PCRs targeting two other genes: *map*1 and pCS20. Amongst the three, the RLB showed the lowest detection rate (i.e. 6.2%), likely to be due to polymorphism of *E. ruminantium* 16S rRNA gene (Faburay *et al*., 2007). Consistently, laboratory studies carried out in the 1970s in Nigeria suggested that several strains of *E. ruminantium* might also be circulating in this country (Leeflang and Ilemobade, 1977a).

A further possible reason explaining the low prevalence detected can be provided by the rigid control practice carried out traditionally by the local Fulani pastoralists, seemingly targeting specifically *A. variegatum* adults (Bayer and Maina, 1984). Considering that this practice is carried out up to three times a week during the wet season (Bayer and Maina, 1984) and that most rickettsial microorganisms require to undergo a 're-activation' of at least 24 hours from the time of the attachment of their vector to vertebrate host (Azad and Beard, 1998), it is possible that this control method might still play a role in reducing the dissemination of the pathogen within herds. Undoubtedly, though, the manual 'de-ticking' of the Fulani, did not affect the detectability of *T. mutans* and *T. velifera*, also transmitted by *A. variegatum* (Uilenberg *et al*., 1974; Young *et al*., 1978). These piroplasms, though, are characterised by higher and longer-lasting parasitaemia in carrier animals (Bishop *et al*., 2009) compared to *E. ruminantium* (Deem *et al*., 1996a).

3.5.12 *Anaplasma platys* – a canine pathogen in cattle

This study provides the first report on the detection of *A. platys* in cattle in Nigeria. Recently, DNA of this rickettsia was found in cattle from Sardinia, Italy (Zobba *et al*., 2014) in cattle ticks from Korea (Kang *et al*., 2013) as well as in sheep

from Senegal (Djiba *et al*., 2013). At present no information is available on the pathogenicity in cattle of *A. platys*, causing a syndrome in dog known as canine cyclic thrombocytopenia (Woody and Hoskins, 1991).

Anaplasma platys is seemingly vectored by *Rhipicephalus sanguineus* Group ticks (Ramos *et al*., 2014), even though an experimental study failed in demonstrating this (Simpson *et al*., 1991). As described in Chapter 2, this tick can be found, although in low burdens, in cattle in this part of Nigeria (Lorusso *et al*., 2013a). Future studies aiming to ascertain this tick-pathogen relation would be desirable, as this might have important implications from a canine health perspective, considering the tight interface between these two animal species in the Fulani herding system (Lorusso *et al*., 2013a).

3.5.13 First detection of *Rickettsia massiliae* in cattle

Interestingly, this study ascertained for the first time the presence of *Rickettsia massiliae* in the whole blood of cattle. Initially isolated in 1990 from *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* ticks in France, this SFG rickettsia was named after the city (i.e. Marseille) where the first description occurred (Beati and Raoult, 1993). At present, its main reservoirs are known to be ticks (e.g. *Rh. sanguineus* and *Rh. turanicus*), in which transstadial and transovarial transmission occur (Matsumoto *et al*., 2005). In very recent times this rickettsia was also found in questing *Rhipicephalus evertsi* ticks, collected from the vegetation in South-Western Nigeria (Reye *et al*., 2012). Interestingly, this species is not present on the Plateau (see Chapter 2 and Lorusso *et al*., 2013a). However in other SSA countries, such as Mali (Parola *et al*., 2001), and Central African Republic (Dupont *et al*., 1994), *R. massiliae* was detected in several *Rhipicephalus* spp. ticks collected from cattle (i.e. *Rhipicephalus muhsamae*, *Rhipicephalus lunulatus*, *Rhipicephalus sulcatus*, *Rhipicephalus turanicus*). All these tick species were found in the survey previously described in Chapter 2 (Lorusso *et al*., 2013a), therefore representing a potential source of infection for the positive animals of this study.

This SFG rickettsia was detected in 7/9 villages, suggesting a widespread distribution within the cattle in the study area. If that was confirmed also for the local

tick fauna of rhipicephalinae, it would raise public health concern, considering the pathogenicity of this species to humans (Vitale *et al*., 2006). With *R. massiliae*, this survey brings to three the species of SFG rickettsiae detected in a very short time in the Plateau State, including also *R. africae*, found in *A. variegatum* from cattle (Ogo *et al*., 2012; Lorusso *et al*., 2013b) and *R. conorii* found in *Rh. sanguineus* ticks from dogs (Kamani *et al*., 2013).

To the best of my knowledge, *R. massiliae* infection has not been previously detected in any livestock species. To date, *R. massiliae* seropositivity was detected, via serum cross-absorption and Western blot, in dogs from California (Beeler *et al*., 2011) and, very recently, *R. massiliae*-Bar29-specific infections were detected in cats from Spain (Segura *et al*., 2014). Therefore, future studies aiming to better address the epidemiology of this rickettsia species in the cattle host would be desirable. Interestingly, the epidemiology of *R. africae* infection was initially thought to be merely dependent on its competent vector and reservoir *Amblyomma variegatum* (Socolovschi *et al*., 2009), until seroconversion was later detected in blood of ruminants from the Caribbean, suggesting a possible involvement of livestock in the life cycle of this SFG rickettsia (Kelly *et al*., 2010).

3.5.14 *Anaplasma bovis*

No *Anaplasma bovis* was detected in this study, although this microorganism, causative agent of a benign form of bovine ehrlichiosis was previously found in Northern Nigeria, after cytological examination of blood smears (Leeflang and Ilemobade, 1977a).

3.5.15 Single TBIs

The large majority (84/92, 91%) of the single infections detected were caused by *T. mutans*, *E*. sp. Omatjenne and *A. marginale*, reflecting the high prevalence of these microorganisms. Examining statistically all infection patterns detected (according to the association screening approach, see also Vaumourin *et al*., 2014), single infections by *E*. sp. Omatjenne (n=30/239, 12.5% of cases of infection with *E*. sp. Omatjenne) and by *R. massiliae* (n=6/19, 31.6% of cases of infection with *R.*

massiliae) were found to be significantly likely ($p<0.001$) to occur in this study (see Table 3.9). This further highlights the need to better understand the pathological importance of *E*. sp. Omatjenne, possibly more feasible in cases of single infections. Moreover, considering the overall low prevalence of *R. massiliae* (i.e. 3.5%), this finding suggests that possibility that the detectability of this rickettsia in the blood stream may be favoured by the absence of other haemoparasites.

3.5.16 Co-infection patterns

Frequency of combinations of co-infective agents (Table 3.8) and results from the statistical analysis of infection patterns through the association screening approach (Vaumourin *et al*. 2014; Table 3.9) are suggestive of lack of competition or antagonist effect among the three *Theileria* species altogether (i.e. *T. mutans*, *T. velifera* and *T. taurotragi*); these and *A. marginale* and *E*. sp. *Omatjenne*; between the latter two species; among the five aforementioned haemoparasites, with or without *B. bigemina* or *A. centrale*. *Anaplasma marginale* and *E*. sp. Omatjenne, the fourth and fifth most frequently detected microorganisms, were found to be likely ($p<0.001$) associated with the three theilerias, only if present together. This may suggest a synergism between these two co-infection patterns (i.e. *A. marginale* + *E*. sp. Omatjenne and *T. mutans* + *T. taurotragi* + *T. velifera*).

Moreover, *T. taurotragi* was always detected in presence of other *Theileria* species. However, the combination of the three theilerias together was found to be a significantly ($p<0.001$) likely type of association, whilst a negative association was found in the co-infections with *T. taurotragi* and *T. mutans* (recorded in 6 samples only) as well as with *T. taurotragi* and *T. velifera* (never detected in this study) (Table 3.9). These results are suggestive of a possible favouring role played by *T. mutans* and *T. velifera*, together, towards the establishment of a parasitaemia by *T. taurotragi*.

In this study, no case of co-existence of the two *Babesia* spp. was recorded in any of the cattle examined (n=704), in accordance with another RLB-based study in 477 cattle in Mozambique (Martins *et al.*, 2010). Besides the aforementioned discrepancy in transmission rates between *B. bigemina* and *B. bovis* by

Rhipicephalus (*Bo*.) ticks, another possible justification for the lack of co-infections detected could be the hypothetical existence of competition between *B. bigemina* and *B. bovis*.

3.5.17 Comparisons of age classes

On the whole, calves had significantly less infection than both juvenile and adult cattle. This is in line with several other studies carried out in SSA (Salih *et al*., 2007), including central-north Nigeria, where a lower proportion of infection (14.5%) was detected in younger cattle compared to adults (36.3%), although an index of quantification of age was not given (Kamani *et al.*, 2010). This is in line with the concept of 'inverse age immunity', with colostral first, and then innate immunity, enabling the low likelihood of appearance of clinical disease in young animals at the time of their peak exposure to a pathogen (Jonsson *et al*., 2012), as discussed for *E. ruminantium* and *B. bigemina* infections, but also with the conditions of endemic stability described for *T. mutans*, *T. taurotragi*, *T. velifera*, and possibly also for *E.* sp. Omatjenne. Importantly, the lower infections rates in calves could also be attributable to the significantly lower tick burden they bear, compared to juvenile and adult cattle, as explained in Chapter 2 (Lorusso *et al*., 2013a).

3.5.18 RLB sensitivity for *A. marginale* detection

The RLB detection limit for *A. marginale* established in this study ranged between 43 and 4.3 copies/µl, depending on whether 1x TE buffer adjusted with bovine genomic DNA or milliQ water were employed, respectively. These sentivities are higher than those described previously employing PCR-based DNA probes (Aboytes-Torres and Buening, 1990; Figueroa *et al*., 1993; Ge *et al*., 1995), RNA probes (Eriks *et al*., 1989), antigen capture enzyme-linked immunosorbent assay (ELISA) (Trueblood *et al*., 1991) and PCR-ELISA tests (Gale *et al*., 1996).

The detection limit obtained in this study was comparable to an infection greater than seven *A. marginale*-infected erythrocytes/µl (dilution series prepared using 1x TE adjusted with bovine genomic DNA) and/or greater than one *A.*

marginale-infected erythrocyte/µl (dilution series prepared using milliQ water). The detection limit recorded using serial dilutions of the purified *A. marginale* 16S plasmid insert in 1x TE buffer adjusted with bovine genomic DNA, was only ten times lower than that obtained using pure water dilutions. Therefore, these results suggest the occurrence of only minimal interference of host DNA in the detactibility of *A. marginale* via RLB, with only a ten fold-difference recorded between pure water and adjusted 1x TE dilutions.

The 1x TE buffer employed was adjusted to a mean concentration of bovine DNA yielded after blood extraction with an overnight digestion (i.e. 29.1 ng/µl), calculated comparing manual and automated methods (Foley *et al.*, 2011). Considering the presence of host DNA in bovine whole blood, it is likely that the detection limit documented preparing serial dilutions with 1x TE adjusted with bovine genomic DNA is closer to the actual sensitivity of the RLB method for the detection of *A. marginale* in fresh whole blood samples.

Nevertheless, a possible interference of the FTA™ card matrix (Whatman BioScience Ltd, Cambridge, UK) employed in the surveys described in Chapter 3 and 4 of this thesis, in the detection threshold of the RLB method is not ruled out and still remains to be addressed.

Original *A. marginale* DNA employed in the present study was obtained by the whole blood of a calf experimentally infected with a Zaria (Kaduna State, Nigeria) stabilate of this microorganism (Zivkovic *et al*., 2007). Considering the very proximate geographic provenance of the original *A. marginale* stabilate and the Plateau State in Nigeria, it is very likely that positive field samples diagnosed with the methodology here described (see Chapters 3 and 4) were infected with the same strain of this haemoparasite.

As Nagore *et al.* (2004a) suggested, a more 'realistic' ('accurate', in their paper) sensitivity value of the overall process could be calculated by diluting and testing infected blood of a known level of parasitaemia. This was actually the approach followed by Molad *et al.* (2006) assessing a sensitivity of 50 infected cells/ml for both *Anaplasma centrale* and *A. marginale*. In the latter study, the RLB sensitivity was determined by tenfold serial dilutions, starting from an initial

concentration of 5×10^9 infected cells, diluted in uninfected erythrocytes. All samples were then adjusted to a final concentration of 5×10^9 cells/ml (Molad *et al*., 2006).

In the study here presented, however, due mainly to geographical and legistlative constraints, it was practically infeasible to use infected fresh bovine blood from the study area as starting material. Moreover, the protocol by Molad *et al*. (2006) not only differed from the present one for the methodology employed in the sensitivity assessment, but also, as already specified, for targeting another fragment within the *Ehrlichia*/*Anaplasma* 16S rRNA gene.

The percentage of infected erythrocytes in the blood stream during *A. marginale* infection varies according to the stage and severity of the infection. During the course of acute infection, *A. marginale* can parasitise 10 to 90% of the circulating erythrocytes, the rickettsemia approximately doubles each day for about 10 days, reaching the order of 10^8–10^9 infected erythrocytes/ml (Kieser *et al*., 1990). Acute anaplasmosis is usually diagnosed based on the history of the animal/herd, the clinical signs and the demonstration of the presence of intra-erythrocytic rickettsiae in the blood smears (OIE, 2012). Importantly, microscopic examination of Giemsa-stained blood smears can only detect levels higher than 10^6 infected erythrocytes/ml, being therefore useful only for diagnosing acute anaplasmosis (Aguirre *et al*., 1988; Gale *et al*., 1996). Blood concentration of *A. marginale*-infected erythrocytes remain microscopically detectable for approximately 20 days (Callow *et al*., 1986), though early diagnosis is essential to enable the correct treatment thus limiting the losses due to acute infection (Aubry and Geale, 2011).

Recovery from acute anaplasmosis is characterized by a persistent life-long infection, with rickettsiaemia levels fluctuating at about 5–6 weeks intervals, ranging from approximately $10^{2.5}$ to 10^7 infected erythrocytes/ml of blood (Kieser *et al*., 1990; Eriks *et al*., 1993; French *et al*., 1998), and reaching a concentration lower than $10^{4.3}$ (i.e. up to $10^{2.5}$) infected erythrocytes/ml for approximately 5 to 8 days for every 5–6 weeks-cycle (Eriks *et al*., 1993). This variation of parasite concentration in the blood is likely related to the emergence of antigenic variants able to escape, at least temporarily, the immune response of the host (McGuire *et al*., 1984; Kieser *et*

al., 1990). Importantly, anaemia persists after the acute phase of the infection, being due to the removal of infected red blood cells by the reticuloendothelial system (OIE, 2012). Its severity therefore depends on the number of parasitized erythrocytes depending, in turn, on the phase of the cycling rickettsiaemia (Eriks *et al*., 1993).

The most 'realistic' sensitivity limit documented in this study (employing the 1x TE dilution series adjusted with bovine genomic DNA), was equivalent to approximately 7×10^3 infected erythrocytes/ml of blood (i.e. equivalent to 7 infected erythrocyte/µl). While this concentration value falls below the parasitaemia of 10^7 and $10^{4.3}$ (i.e. 19,953) infected erythrocytes/ml, recorded during part of the cycles of chronic infections, it is still above the lowest rickettsiaemia of $10^{2.5}$ (i.e. 316) infected cells/ml, seemingly occurring for 5 to 8 days, every 5–6 weeks, in chronically infected cattle (Eriks *et al*., 1993). This finding, therefore, highlights the capacity of the RLB test in detecing chronic and sub-clinical *A. marginale* infections for most of their duration, except for a period of approximately a week (i.e. 5–8 days), occurring every 5–6 weeks, when the rickettsiamiae reaches the value of $10^{2.5}$ infected erythrocytes/ml of blood (Eriks *et al*., 1989). This is of great epidemiological relevance, considering that chronically infected cattle may still act as reservoir of the infection for arthropod vectors (Zaugg *et al*., 1986).

As peristently infected carriers usually maintain high antibody titres, tests traditionally employed for the detection of chronic infections include serological assays, such as a competitive enzyme linked immunosorbent assay (cELISA), card agglutination test (CAT), complement fixation (CF) test and IFAT (reviewed in OIE, 2012). However, it should be noted that serological diagnosis can be affected by cross-reactivity between *A. marginale* and *A. centrale* as well as between *A. marginale* and *Anaplasma phagocytophilum* and several *Ehrlichia* spp. (Al-Adhami *et al*., 2011; Dreher *et al*., 2005). Moreover, the CAT is characterized by lack of specificity and risk of subjective interpretation of results (OIE, 2012). In addition, the antigenic suspension employed in this assay, can be difficult to prepare, and it may vary from batch to batch and according to the laboratory of manufacturing (OIE, 2012). The CF test has been used extensively for many years; however it shows variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor reproducibility (OIE, 2012).

Therefore, molecular tools should be considered as the most appropriate tests currently available for detecting carrier infections by *A. marginale* and they may also be employed to confirm cases of acute anaplasmosis.

A nested PCR was developed to detect *A. marginale* infections in carrier cattle, being able to identify as few as 30 infected erythrocytes/ml of blood, well below the sensitivity assessed in the present study, as well as the lowest levels of rickettsiaemia recorded in carrier cattle (Torioni de Echaide *et al*., 1998). However, consisting of two consecutive amplifications, nested PCR poses non negligeable quality control and specificity issues (Torioni de Echaide *et al*., 1998).

Recently, an iTaq™ real-time PCR assay was developed in Italy (Carelli *et al*., 2007), targeting a 16S rRNA gene fragment previously identified by Molad *et al*. (2006), showing a higher sensitivity (i.e. 10 copies of standard DNA/µl and 30 *Anaplasma*-infected erythrocytes/ml) than that of the RLB assay here assessed.

While real time-PCR presents the advantage of using a single closed tube for amplification, reducing the opportunities for amplicon contamination, the equipment needed for its implementation is expensive and may be beyond the capacity of some laboratories, especially in developing countries. Conversely, the RLB also offers the great advantage of enabling the screening of one sample for multiple species and genera of microorganisms at the same time. This represents a great added value of this method, especially useful in contexts of co-endemicity by several TBIs as the tropical regions.

3.6 Conclusion

This study disclosed the occurrence of numerous bovine tick-borne haemoparasites in Nigeria, including agents of veterinary and zoonotic concern. The RLB proved to be suitable for broad scale epidemological surveys like the present, enabling the detection of carrier states and multiple infections. Endemic stability for *T. mutans, T. velifera, T. taurotragi, A. marginale, E.* sp. Omatjenne and possibly *B. bigemina* is postulated.

As elucidated in Chapter 1 (see also Section 1.2.3), based on the existing literature on TBIs, to establish in the field, endemic stability requires i) high prevalence of infection in the vector tick; ii) the presence of a durable vertebrate reservoir representing a steady source of infection for competent ticks; iii) high resistance to the emergence of clinical disease in the vertebrate host, especially at a young age (Uilenberg, 1995; Deem *et al.*, 1996a; Jonsson *et al.*, 2012). The survey here presented ascertained that conditions ii) and iii) are satisfied in the study area at least for the most prevalent microorganisms detected, namely *T. mutans, T. velifera, T. taurotragi, A. marginale* and *E.* sp. Omatjenne, and possibly also for *B. bigemina*, whose lower overall prevalence may be attributable to sensivity limitations of the RLB assay. At present, very little information is available on the infection rates of ticks from Central Nigeria, with regards to the pathogens investigated in this study. It is therefore difficult to assess whether the aforementioned condition i) (i.e. 'high prevalence of infection in the vector tick'), is also met in the study area. In the only study carried out to date based on the molecular processing of ticks collected from cattle in Central Nigeria (i.e. Plateau and Nassarawa States), only two tick species were screened, namely *A. variegatum* (n=153) and *Rh.* (*Bo.*) *decoloratus* (n=45) (Ogo *et al.*, 2012). Results highlight modest prevalence of infections for *A. variegatum*, for which the most prevalent microorganism was the zoonotic *Rickettsia africae* (7.8%), followed by *Babesia* spp. (3.9%), *B. bigemina* (1.3%) and *B. divergens* (0.6%) (Ogo *et al.*, 2012). Interestingly, *T. mutans, T. velifera* and *E. ruminantium* were not detected in any of the *A. variegatum* screened (Ogo *et al.*, 2012). Nonetheless, the screening of *Rh.* (*Bo.*) *decoloratus* ticks revealed a rather high prevalence of *A. marginale* (20%), followed by *R. africae* (4.4%) (Ogo *et al.*,

2012). These findings suggest that the contribution of ticks' infection rates to the establishment of endemic stability may vary according to the pathogen species and the number of vectors contributing to their transmission. For example, in the case of *T. mutans* and *T. velifera*, transmitted by only one tick species (i.e. *A. variegatum*), it is possible that the carrier status and the resistance to clinical disease of the cattle host may play a major role in the maintenance of endemic stability, compared to the infection rates of ticks, in the Plateau State. Conversely, considering the prevalence of 20% of *A. marginale* detected in *Rh.* (*Bo.*) *decoloratus* (Ogo *et al*., 2012), and the fact that other tick species (e.g. *Rh.* (*Bo.*) *annulatus* and *Rh. simus* Group) and blood-sucking flies (e.g. *Stomoxys* spp., *Tabanus* spp., *Chrysops* spp. and *Haematobia* spp.), may act as vectors of this rickettsia (Aubry and Geale, 2011), the contribution of arthropods' infection rates may not be negligible for the establishment and maintenance of endemic stability of this pathogen in the study area. As further stressed in Section 5.2.1, screening of a larger number of ticks from a variety of tick species, including also specimens collected from the environment may help address this issue.

In this study, a 'less stable' epidemiological scenario is suggested for *A. centrale*, whose occurrence in Central Nigeria was confirmed on a molecular basis.

Similarly, this study ascertained the presence of *B. bovis* in cattle in Central Nigeria. The epidemiology of this infection in Nigeria needs to be thoroughly investigated, to better understand its competent vector in the area.

The few cases of *E. ruminantium* infection, detected only in juvenile and adult cattle, in absence of clinical signs referable to heartwater, are suggestive of past infections.

This work also broadens the knowledge on the distribution of *T. taurotragi* in SSA, providing the first evidence of the detection of this piroplasm DNA in West Africa. Future endeavours aiming to better understand the epidemiology (i.e. vector as well as vertebrate reservoirs) of this *Theileria* species would be very desirable from future studies.

New host-pathogen associations were also detected. This study provides the first record of bacteraemia by *R. massiliae* in cattle, as well as the first report of the detection of *E.* sp. Omatjenne and *A. platys* in cattle in Nigeria.

Furthermore, the RLB method proved to be suitable for the identification of most 'carrier' and sub-clinical infections by *A. marginale*.

Chapter 4 – Effect of 0.005% deltamethrin restricted application protocol (RAP) on bovine tick-borne infection kinetics in Central Nigeria – A longitudinal field study

4.1 Introduction

Together with TBIs, tsetse-borne African animal tryopanosomiasis (AAT) represents a steady threat to cattle health and productivity in several SSA African countries (Ilemobade, 2009) including Nigeria (Kirby, 1963; Maclennan, 1963; Killick-Kendrick and Godfrey, 1963; Hill *et al*., 1976; Opasina and Ekweruke, 1987; Nawathe *et al*., 1988; Wosu and Nwanta, 1989; Daniel *et al*., 1993; Kalu, 1996a,b; Kalu and Lawami, 1996; Enwezor *et al*., 2009, 2012).

As indicated in Chapter 1, in May 2010, a four-year research-for-development initiative entitled 'Stamp Out *Sammore* (SOS)' was launched in Central Nigeria with the aim to specifically tackle AAT on the Plateau. Funded by the UK's Biotechnology and Biological Sciences Research Council (BBRSC) under the 'Combating Infectious Diseases in Livestock for International Development (CIDLID)' awarding scheme, the programme was carried out under the umbrella of the Nigerian Institute for Trypanosomiasis Research (NITR) in partnership with the University of Edinburgh and the French pharmaceutical multinational company CEVA Santé Animale (which donated drugs for vector control). The project's award mainly derived from work carried out recently in the Plateau State disclosing an alarmingly high prevalence of AAT (i.e. 46.8%, 95% CI: 39.0–54.5%; Majekodunmi *et al*., 2013).

The SOS programme targetted a number (i.e. six) of villages of the Plateau State, where a set of trypanosomiasis-focused interventions were rolled out, aspiring to improve the health and productivity of the Fulani cattle and, ultimately, the livelihoods of the pastoralist communities attached to them. Amongst others, a trial based on the restricted application of 0.005% deltamethrin (Vectocid®, CEVA Santé Animale, Libourne, France) in spray formulation targeted part of the herds in each village.

Belonging to the second generation of synthetic pyrethroids emerging in the early 1970s (Elliot *et al*., 1974), deltamethrin is one of the most satisfactory compounds within its class, being photostable, of rather limited toxicity for vertebrates (Elliot *et al.*, 1973) and effective at low dosage against a broad variety of pests (Zerba, 1988). Deltamethrin acts as both an acaricide and insecticide, rapidly

causing shock (the so-called 'knock-down effect') in arthropods, due to the opening of the Sodium (Na^+) channels, leading to the depolarization of the nervous cells in the cerebral ganglia (Narahashi, 1982). These two effects make the use of this compound advantageous in vector control: 'residual activity' (i.e. effectiveness lasting for several weeks, depending on the concentration of the compound) (Beugnet and Franc, 2012), and repellency, consisting in the repulsion of the ectoparasites from the skin of the treating host, preventing them attaching and/or feeding (Halos *et al*., 2012).

Initially employed to repel ticks (Young *et al*., 1988), deltamethrin soon started being used in the African livestock sector also to repel tsetse flies (Thomson and Wilson, 1992), in several formulations (i.e. spot-on, pour-on, spray, dips, impregnated targets) (Chizyuka and Luguru, 1986; Thomson, 1987; Thompson *et al*., 1991; Bauer *et al*., 1992; 1995; Fox *et al*., 1993; Luguru *et al*., 1993; Okello-Onen *et al*., 1994; Vale *et al*., 1999; Van de Bossche and Mudenge, 1999; Warnes et al., 1999; Magona *et al*., 2000; Rowlands *et al*., 2001; Okiria *et al*., 2002; Torr *et al*., 2007; Bouyer *et al*., 2007, 2009; Bekele *et al*., 2010), allowing, in some instances, an 'integrated' control of several bovine vector-borne diseases in SSA (Bauer *et al*., 1992; Fox *et al*., 1993; Van de Bossche and Mudenge, 1999).

However, routine treatment of cattle with pyrethroids can, in the long term, lead to the development of resistance in ticks (e.g. sub-genus *Boophilus* spp.) (Bruce and Wilson, 1998) and it may also cause the disruption of TBIs endemic stability (Eisler *et al*., 2003), together with a reduction of the invertebrate fauna involved in the decomposition of cattle dung (Vale and Grant, 2002; Vale *et al*., 2004).

Recently a protocol based on the application of deltamethrin only to the lower quarters (i.e. legs, brisk, abdomen and groin) and the neck of cattle has proven successful in halting bovine AAT in several African countries such as Zimbabwe (Torr *et al*., 2007), Burkina Faso (Bouyer *et al*., 2007; 2009) and Uganda (www.stampoutsleepingsickness.com) and has received increasing attention. This 'restricted application protocol' (RAP) was developed based on the evidence that at least two of the principal vectors of trypanosomes in SSA (i.e. *Glossina pallidipes* and *Glossina morsitans morsitans*) feed mainly on the abdomen and lower limbs area of older and larger cattle (Torr and Hargrove, 1998; Vale *et al*., 1999; Torr *et al*.,

2001). Besides its effectiveness against trypanosomiasis, this method is claimed to be also advantageous for being environmentally and economically sustainable, allowing the use of lower volumes of pyrethroid (Torr *et al*., 2007) and reducing the risk of alteration of the dung fauna (Vale and Grant, 2002).

It is proposed that RAP can be successfully put into practice without altering the effectiveness of deltamethrin against AAT. Considering that tsetses are more sensitive to pyrethroids than ticks, endemic stability for certain TBIs is retained by adopting a set of measures such as: i) avoiding treating calves and ii) reducing the frequency of treatment (e.g. no more often than once per month) (Eisler *et al*., 2003).

Furthermore, some authors also argue that RAP does not interfere with the preferential sites of attachments of ticks, assuming that they differ from those of tsetse flies (Eisler *et al*., 2003; Torr *et al*., 2007). However, Chapter 1 and 2 of this thesis suggest that this may not be the case for most tick species infesting cattle in Nigeria, which are usually found attached to body areas within the lower quarters of the animals, including abdomen, udder, and groin (*Amblyomma variegatum*, *Hyalomma* spp.); dewlap (*Rhipicephalus* (*Boophilus*) spp.); axilla (*A. variegatum*, *Rhipicephalus* (*Bo.*) spp.) and, though to a minor extent, the upper (immature stages, *Rhipicephalus* (*Bo.*) spp.) and the lower part of the limbs (*Hyalomma* spp.) too.

Nonetheless, amongst the numerous trials carried out to date to assess the efficacy of different concentrations (i.e. 0.00375%; 0.005%; 0.4%; 1%) and formulations (e.g. spray, pour-on, etc.) of deltamethrin in controlling tsetse infestation and/or bovine AAT in several African countries (e.g. Burkina Faso, Ethiopia, Tanzania, Uganda, Zambia and Zimbabwe) (Chizyuka and Luguru, 1986; Thomson, 1987; Thompson *et al*., 1991; Bauer *et al*., 1992; 1995; Fox *et al*., 1993; Luguru *et al*., 1993; Okello-Onen *et al*., 1994; Vale et al., 1999; Van de Bossche and Mudenge, 1999; Warnes *et al*., 1999; Magona *et al*., 2000; Rowlands *et al*., 2001; Okiria *et al*., 2002; Torr *et al*., 2007; Bouyer *et al*., 2007, 2009; Bekele *et al*., 2010), to date only a few studies have evaluated the effect of such treatment protocols either on tick burdens (Okello-Onen *et al*., 1994; Stachurski and Lacelot, 2006) or on the epidemiology of a single TBI (i.e. babesiosis) by means of serology (Van de Bossche and Mudenge, 1999).

4.2 Aims

Therefore, the present study aimed to assess the effect of a monthly application of 0.005% deltamethrin in spray formulation, applied only to the lower quarters of cattle according to a RAP, on the kinetics of bovine TBIs in the Plateau State, Central Nigeria. To do so, an 11-month long longitudinal survey was implemented, with the same animals, from a treated and control group, being sampled every three months after baseline and screened by means of molecular tools for a broad array of TBIs.

Results generated would help orientate the development of future control strategies to be rolled out across SSA, aiming to tackle tsetse-borne trypanosomiasis in TBIs-endemic areas.

4.3 Materials and Methods

This study was conceived concertedly by members of the Welburn Research Group, Division of Pathway Medicine, School of Biomedical Sciences, The University of Edinburgh, UK (e.g V. Lorusso[9], A. Majekodunmi[10] and S. Welburn[11]) and the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos, Plateau State, Nigeria (e.g. C. Dongkum[12] and A. Igweh[13]).

All field operations including blood sampling and treatment procedures (i.e. diminazene injections at two intervals and monthly 100% RAP applications) were carried out by staff personnel from NITR. The author of this thesis was responsible for the molecular processing of samples, statistical analysis and interpretation of results, and also contributed to the design of the study.

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4.3.1 Timeline of activities

The field study was carried out from March 2012 to February 2013, as illustrated by Table 4.1. Samples were screened in September and October 2013.

Table 4.1 – Timeline of events of the field trial.

Treated group	Time of the year	Control group
- Animal enrolment - ***Baseline***	MARCH 2012	- Animal enrolment - ***Baseline***
-Diminazine curative treatment (2 week interval) - ***Follow up I*** - 100% RAP	Early APRIL Late APRIL	-Diminazine curative treatment (2 week interval) - ***Follow up I***
- 100% RAP	MAY	
- 100% RAP	JUNE	
- 100% RAP	JULY	
- Follow up II - 100% RAP	AUGUST	***- Follow up II***
- 100% RAP	SEPTEMBER	
- 100% RAP	OCTOBER	
- Follow up III	NOVEMBER	***- Follow up III***
- 100% RAP	DECEMBER	
- 100% RAP	JANUARY 2013	
- Follow up IV	FEBRUARY 2013	***- Follow up IV***

4.3.2 Study area

The initial tsetse borne-AAT-focused intervention targeted six villages (i.e. Bokkos, Daffo, Hurti, Maiyanga, Mbar, Tambes), belonging to three neighbouring LGAs in the central part of the Plateau State, namely Bokkos (villages of Bokkos, Daffo, Hurti, Maiyanga, Mbar), Pankshin (village of Tambes; see also Figures 2.2 and 3.1). These villages were identified as representative of the entire study area not only for their agro-ecological characteristics, as well as from a livestock management standpoint, but also for their prevalences of AAT being representative for the whole of the Plateau State previously surveyed (Majekodunmi, 2011; Majekodunmi *et al*., 2013).

All villages are located within the North Savanna vegetation zone, in the sub-humid region of Nigeria, where the dry season generally extends from November to

April, and the wet season from April-May to October, with most (approximately 80%) of the rains occurring between June and September (Odumodu, 1983).

4.3.3 Animal enrolment

In March 2012, six household's herds were selected in each village, to be subjected to different treatment regimens, as follows:

- Group A: consisting of 80 cattle from the same herd, sprayed monthly with 0.005% deltamethrin (i.e. Vectocid®, CEVA Santé Animale, Libourne, France), according to a RAP, applied to 100% (n=80/80) of the animals of the herd;

- Group B: 80 cattle from the same herd, left untreated without arthropocides throughout the length of the study;

- Group C: 80 cattle from the same herd administered monthly a pour-on formulation of deltamethrin;

- Group D: 80 cattle from the same herd, 50% of which (n=40/80) sprayed monthly with 0.005% deltamethrin, according to RAP;

- Group E: 80 cattle from the same herd, 25% of which (n=20/80) sprayed monthly with 0.005% deltamethrin, according to RAP;

- Group F: 80 cattle from the same herd, injected every three months with a preventive dose (0.5–1 mg/kg of live body weight, LBW) of isometamidium solution (i.e. Veridium®, CEVA Santé Animale, Libourne, France), for a total of five treatment times.

All animals enrolled in the study were ear-tagged with an identification number ranging from 1 to 80. Each animal's identification data (i.e. ear-tag number, breed, sex, coat colour, and age at enrolment time) were recorded in individual files. Consistent with the work presented in Chapters 2 and 3, the age of the cattle was estimated using the dentition score method developed for zebu cattle under a low plane of nutrition (Kikule, 1953) and by asking their owners the approximate date of birth of each of their animals. All animals enrolled in the study were older than six months, according to the recommendations by Torr *et al.* (2007) in order to preserve TBIs endemic stability, when occurring (see Section 3.5 of this thesis for indications on this study area).

Throughout the study period, however, several animals were lost, mainly due to either i) sales, ii) loss of ear-tags during the grazing; iii) reluctance of their owners in keeping their animals in the study and/or identifiable with an ear-tag. Therefore, in each of the study groups in all six villages, a number lower than 80 animals could be longitudinally followed up throughout the entire duration of the trial (see Table 4.2).

Table 4.2 – Study villages and allocated groups including animals that could be followed up longitudinally from March 2012 to February 2013. Highlighted cells refer to animals object of the study of this chapter.

Villages	100% RAP (Group A)	Control (Group B)	50% RAP (Group C)	25% RAP (Group D)	Deltamethrin pour on (Group E)	Isometamidium (Group F)	Total
Bokkos	0	26	1	20	59	20	**126**
Daffo	16	31	0	0	7	4	**58**
Hurti	43	63	15	0	0	0	**121**
Maiyanga	26	8	53	48	29	21	**185**
Mbar	16	0	0	0	3	0	**19**
Tambes	22	0	0	0	8	53	**83**
Total	**123**	**128**	**69**	**68**	**106**	**98**	**592**

The present chapter will therefore only examine animals from Group A and Group B from the village of Hurti (Bokkos LGA), where the largest number of animals subjected to 100% RAP as well as those left untreated (i.e. 'control' group), could be monitored until the end of the study period. Hence, the following paragraphs of this Chapter will only refer to Group A and B of the aforementioned village.

4.3.4 Baseline sampling

Immediately after having been identified with ear-tags and informative data in individual sheets, all enrolled animals were blood-sampled via venipuncture of the jugular vein, according to the same protocol described in Chapter 3. Briefly, approximately 100 µl of whole blood collected from each animal was applied onto an FTA™ card (Whatman BioScience Ltd, Cambridge, UK). After being left to air-

dry over night at room temperature, all samples were placed in foil pouches with a silica desiccant and mailed to the University of Edinburgh to be subjected to molecular processing.

4.3.5 Diminazene treatment

After the baseline sampling, in early April 2012, all enrolled animals were subjected to an intramuscular (IM) injectable treatment with diminazene aceturate (Veriben®, CEVA Santé Animale, Libourne, France) at the dosage prescribed by the manufacturer (i.e. 3.5 mg/kg of LBW), in order to clear them from trypanozoon infection. The same treatment was repeated two weeks later (i.e. mid April 2012) to ensure the accomplishment of its effectiveness. Thereafter, and for the rest of the study period, all animals from both Group A and B were left untreated with regards to any systemic medicament (e.g. antibiotic, trypanocidal, etc.).

However, considering that diminazene acts also as a babesicidial (reviewed by Bock *et al*., 2004), it was expected that this treatment regimen would also affect possible infections by *Babesia* spp.

4.3.6 Post curative treatment sampling

In order to assess the efficacy of the diminazene-based treatment, all animals were blood-sampled at the end of April 2012, according to the methodologies described in Section 3.3.3.

4.3.7 Deltamethrin treatment

From April 2012 to February 2013, all animals from Group A were sprayed monthly with 0.005% deltamethrin (Vectocid®, CEVA Santé Animale, Libourne, France) applied only to the lower quarters (i.e. abdomen and limbs) and the neck of each cattle according to the RAP described by Torr *et al.* (2007) (Figure 4.1). Each monthly RAP took place in the same site of the village, not accessible to animals of Group B throughout the entire duration of the study.

Figure 4.1 – Cattle being sprayed with 0.005% deltamethrin applied only to the lower quarters and the neck (© Felix Agyambal).

4.3.8 Follow up sampling

Further to the sampling of late April 2012, every three months, until February 2013, all animals of both Group A and B were blood-sampled as previously described (see Section 3.3.3). After having been collected and air-dried on FTA™ cards, all samples were shipped to the University of Edinburgh for further molecular processing, kept in sealed envelopes with silica desiccant.

4.3.9 Molecular proceedings

Once in the laboratory at the University of Edinburgh, each sample was subjected to a molecular processing consisting of: i) DNA purification; ii) three PCR amplifications targeting the *Ehrlichia*/*Anaplasma* 16S, the *Theileria*/*Babesia* 18S and the *Rickettsia* spp. 16S rRNA genes followed by iii) RLB and iv) visualization of results via x-ray development of hypersensitive films (Amersham, UK) according to the methodologies described in Chapter 3 of the present thesis. Differently from the protocol previously described, *Theileria equi* DNA provided by the Utrecht Centre for Tick-borne Diseases (UCTD) was used as positive control for the *Theileria*/*Babesia* 18S PCR, as well as for the RLB.

Further to the experiments described in Chapter 3 and 4, a newly designed Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.) was employed in this study, including a larger number of 'catch-all' and species-specific oligonucleotide probes (working concentration: 400µM) containing a N-terminal N-(trifluoroacetamidohexyl-cyanoethyle, N,N-diisopropyl phosphoramidite [TFA])-C6 amino liker (Isogen, The Netherlands), enabling each sample to be screened for an array of up to 5 different genera and 25 species of possible bovine tick-borne microorganisms simultaneously. The reasons for the expansion of the initial library of the oligonucleotide sequences employed in the survey described in Chapter 3, were essentially:

a) Findings of Chapter 3 (e.g. unexpected low prevalence of *E. ruminantium*; detection of *A. platys* and SFG rickettsiae in the bovine blood);
b) Reports featuring in press during the time inter-curing between the survey previously described and the initiation of the present study, highlighting the occurrence of several SFG rickettsiae in Nigeria (e.g. Ogo *et al*., 2012; Reye *et al*., 2012);
c) Suggestions by peer-scientists, outcome of idea exchanges taking place at symposia, conferences (i.e. 7th Ticks and Tick-borne Pathogens Conference, Zaragoza, Spain; 1st Regional Meeting of the Society of Tropical Veterinary Medicine, Phuket, Thailand), and training courses (i.e. 1st RLB 'Summer

Course', Utrecht University, Utrecht, The Netherlands) in the years 2011 and 2012.

When available, sequences referring to several strains of the same microorganisms (i.e. *Anaplasma phagocytophilum*: 1–4; *Babesia caballi*: 1–3; *Theileria equi*/*Theileria equi*-like) were covalently linked to the RLB membrane, prepared according to the methodologies described in Chapter 3. With special reference to *E. ruminantium*, in the light of the results elucidated in Chapter 3, an additional probe complementary to another fragment of the 16S hypervariable gene region amplified by the PCR primers, was chosen, with the aim to possibly increase the chances of detection of this pathogen (see Table 5.3).

Table 4.3 – 'Catch-all' and species-specific oligonucleotide probes covalently linked to the RLB membrane employed in this study.

	Tick-borne Microorganism's Genera/Species	**Probe Sequence (from 5'–3')**	**Tm (ºC)**	**Reference**
1	*Ehrlichia/ Anaplasma* catch-all	GGGGGAAAGATTTATCGCTA	58	Bekker *et al.* (2002)
2	*Anaplasma bovis*	GTAGCTTGCTATG(A/G)GAACA	56–58	Georges *et al.* (2001)
3	*Anaplasma centrale*	TCGAACGGACCATACGC	61	Bekker *et al.* (2002)
4	*Anaplasma marginale*	GACCGTATACGCAGCTTG	59	Bekker *et al.* (2002)
5	*Anaplasma phagocytophilum* – 1	TTGCTATAAAGAATAATTAGTGG	54	Schouls *et al.* (1999)
6	*Anaplasma phagocytophilum* – 2	TTGCTATGAAGAATAATTAGTGG	56	Schouls *et al.* (1999)
7	*Anaplasma phagocytophilum* – 3	TTGCTATAAAGAATAGTTAGTGG	55	Schouls *et al.* (1999)
8	*Anaplasma phagocytophilum* – 4	TTGCTATAGAGAATAGTTAGTGG	56	Schouls *et al.* (1999)
9	*Anaplasma platys*	GTCGTAGCTTGCTATGATA	55	Unpublished
10	*Ehrlichia ruminantium* – 1	AGTATCTGTTAGTGGCAG	54	Bekker *et al.* (2002)
11	*Ehrlichia ruminantium* – 2 (i.e. BAA015)	ATTTCTAATAGCTATTCCAT	50	Alsopp et *al.* (1999)
12	*Ehrlichia* sp. Omatjenne	CGGATTTTTATCATAGCTTGC	57	Bekker *et al.* (2002)
13	*Ehrlichia chaffeensis*	ACCTTTTGGTTATAAATAATTGTT	55	Schiuks *et al.* (1999)
14	*Theileria/Babesia* catch-all	TAATGGTTAATAGGA(A/G)C(A/G)GTTG	55–59	Matjila *et al.* (2008a)

	Tick-borne Microorganism's Genera/Species	Probe Sequence (from 5'–3')	Tm (ºC)	Reference
15	*Babesia* catch-all 1	ATTAGAGTGTTTCAAGCAGAC	57	Nijhof (unpublished)
16	*Babesia* catch-all 2	ACTAGAGTGTTTCAAACAGGC	60	Nijhof (unpublished)
17	*Babesia bigemina*	CGTTTTTTCCCTTTTGTTGG	58	Gubbels *et al*. (1999)
18	*Babesia bovis*	CAGGTTTCGCCTGTATAATTGAG	61	Gubbels *et al*. (1999)
19	*Babesia caballi* – 1	GTGTTTATCGCAGACTTTTGT	59	Butler *et al*. (2008)
20	*Babesia caballi* – 2	GTTGCGTT(G/T)TTCTTGCTTTT	58–60	Govender *et al*. (2011)
21	*Babesia divergens*	ACT(A/G)ATGTCGAGATTGCAC	57–59	Oosthuizen *et al*. (2009)
22	*Theileria* catch-all	ATTAGAGTGCTCAAAGCAGGC	62	Matijla *et al*. (2008c)
23	*Theileria annulata*	CCTCTGGGGTCTGTGCA	62	Georges *et al*. (2001)
24	*Theileria buffeli*	GGCTTATTTCGG(A/T)TTGATTTT	56–57	Gubbels *et al*. (1999)
25	*Theileria equi*	TTCGTTGACTGC(C/T)TTGG	56–59	Butler *et al*. (2008)
26	*Theileria equi*-like	TTCGTTGTGGCTTAGTTGGG	62	Unpublished
27	*Theileria mutans*	CTTGCGTCTCCGAATGTT	59	Gubbels *et al*. (1999)
28	*Theileria parva*	GGACGGAGTTCGCTTTG	60	Nijhof *et al*. (2003)
29	*Theileria taurotragi*	TCTTGGCACGTGGCTTTT	62	Gubbels *et al*. (1999)
30	*Theileria velifera*	CCTATTCTCCTTTACGAGT	54	Gubbels *et al*. (1999)
31	*Theileria* sp. MSD4	GCTTATTTCGGCGACCTC	60	Unpublished
32	*Theileria* sp. (duiker)	CATTTTGGTTATTGCATTGTGG	59	Nijhof *et al*. (2005)
33	*Rickettsia* catch-all	TTTAGAAATAAAAGCTAATACCG	54	Christova *et al*. (2003)
34	*Rickettsia conorii*	CTTGCTCCAGTTAGTTAGT	55	Christova *et al*. (2003)
35	*Rickettsia helvetica*	GCTAATACCATATATTCTCTATG	53	Christova *et al*. (2003)
36	*Rickettsia massiliae*	TGGGGCTTGCTCTAATTAGT	60	Hornok *et al*. (2013)
37	*Rickettsia sp.* (DnS14)/*raoultii*	CTAATACCGCATATTCTCTACG	57	Nijhof *et al*. (2007)

*Tm = melting temperature.

4.3.10 Statistical analysis

Differences between Group A and B with regards to the prevalence of each tick-borne haemoparasite at each sampling time were calculated using the two-tailed Fisher's exact test with the WinPepi software. P values lower than 0.05 were considered as statistically significant. For *A. centrale*, *B. bigemina*, *B. bovis*, and *Ehrlichia*/*Anaplasma* 'catch-all' only positive samples, the cumulative incidence (CI) was calculated, from follow up I (April 2012) to IV (February 2013) according to the formula:

$$CI = \frac{\text{No of individuals infected over a period}}{\text{No of uninfected individuals in the population at the beginning of that period}}$$

4.4 Results

4.4.1 Study animals

The 43 animals of Group A that could be monitored throughout the entire study included one juvenile (age: 1 and a half years) and 42 adult (age: ≥ 2 years) cattle, whereas the 63 animals followed up in Group B comprised 31 juvenile and 32 adult cattle.

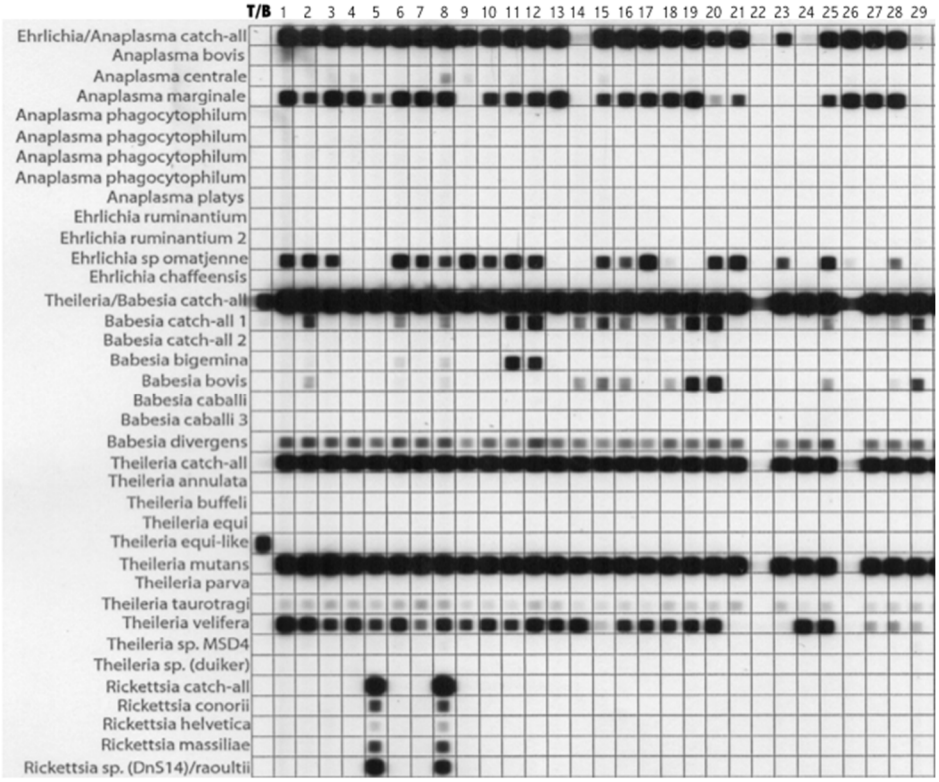
4.4.2 Overall Infection rates

At baseline, all animals (100%) were infected in both groups A and B. The prevalence of positive animals remained rather steady throughout the entire duration of the study, ranging between 98 and 100% in both groups (see Table 4.4). In particular, in Group A, only one animal was found negative in the month of August 2012 and another in the month of February 2013. In Group B one animal was found not infected in the month of April and another one in the month of November 2012.

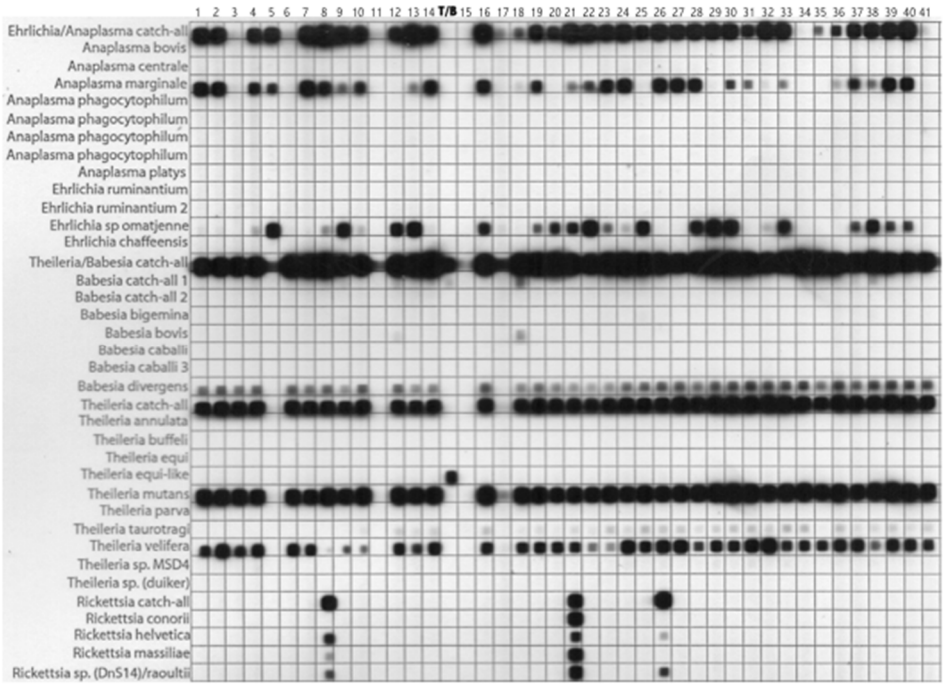
Table 4.4 – Number of infected animals compared to total sample size in treated and control group throughout the study period.

	Infected/Total animals (%)				
	March 2012	**April 2012**	**August 2012**	**November 2012**	**February 2013**
Group A (treated)	43/43 (100%)	43/43 100%)	42/43 (97.7%)	43/43 (100%)	42/43 (97.7%)
Group B (control)	63/63 (100%)	62/63 (98.4%)	63/63 (100%)	62/63 (98.4%)	63/63 (100%)

Apart from one case of single infection (by *Theileria mutans*) detected in one animal from Group B in April 2012, all sampled cattle were found infected by at least two or more tick-borne microorganisms for the entire duration of the study. An example of representative sets of RLB results for both Group A and B is provided by Figure 4.2 (a–b).



(a)



(b)

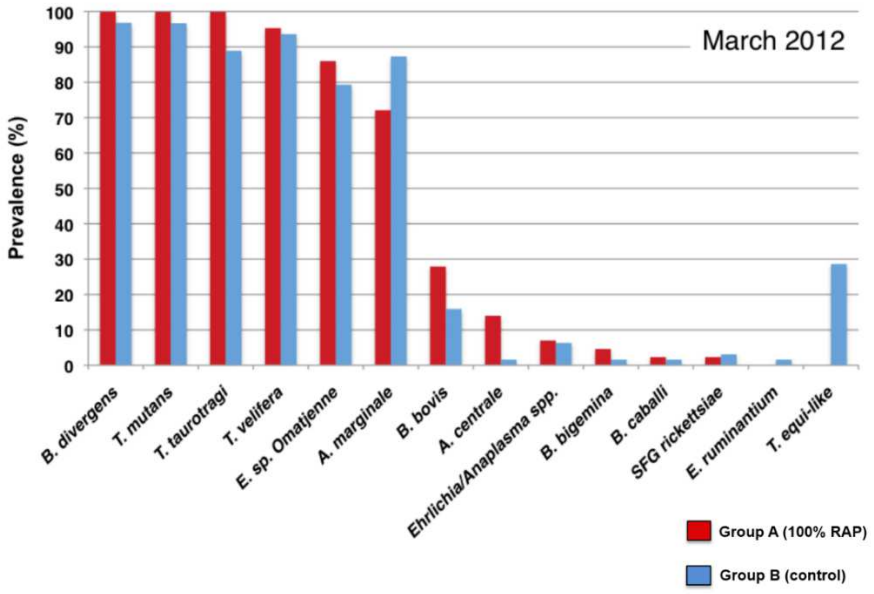
Figure 4.2 – Examples of RLB developments obtained.

(a) = August 2012 (follow up II), Group A [samples: 1–29, 'T/B': *Theileria*/*Babesia* positive control (i.e. *T. equi*); note the high number of *B. bovis* positive signals]; **(b) =** November 2012 (follow up III), Group B [samples 1–41, 'T/B': *Theileria*/*Babesia* positive control (i.e. *T. equi*)].

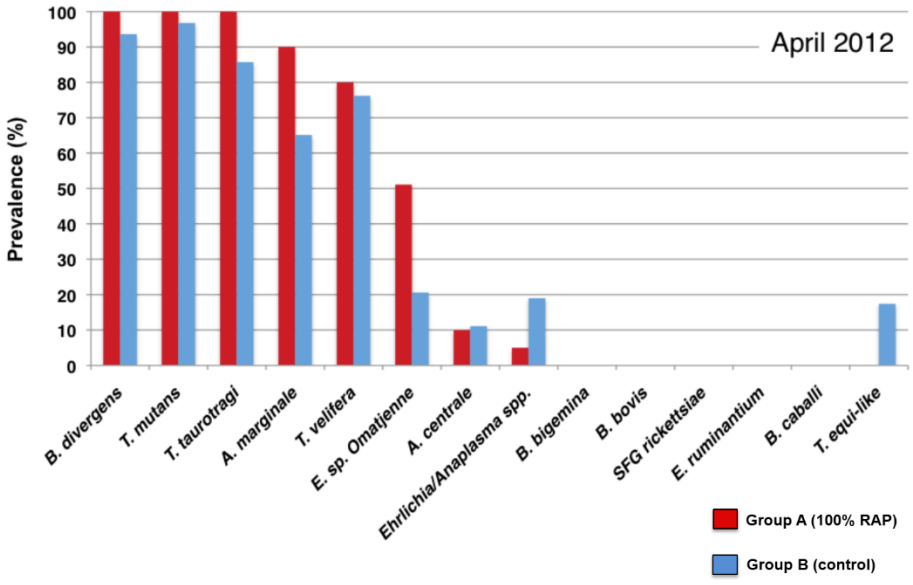
Overall, 13 different tick-borne microorganisms were detected in both groups, namely *Anaplasma centrale*, *Anaplasma marginale*; *Ehrlichia* species Omatjenne, *Ehrlichia ruminantium; Babesia bigemina*, *Babesia bovis*, *Babesia caballi*, *Babesia divergens*; *T. mutans*, *Theileria taurotragi*, *Theileria velifera*, *Theileria equi*-like; *Rickettsia* spp. (of the spotted fever group, SFG). Moreover, in both groups A and B, a number of animals resulted positive for the *Ehrlichia*/*Anaplasma* 'catch-all' probe only, not followed by any species-specific *Ehrlichia* or *Anaplasma* detection, throughout the entire length of the study.

Prevalence of each tick-borne haemoparasite detected at each sampling time of this study is illustrated in Figure 4.3 (a–e) for both Group A and B. Throughout the trial, for both Group A and B, the six most prevalent (>50% of animals infected) microorganisms included *T. mutans, T. velifera, T. taurotragi, B. divergens, A. marginale* and *E.* sp. Omatjenne. Other haemoparasites, such as *B. bigemina, B.*

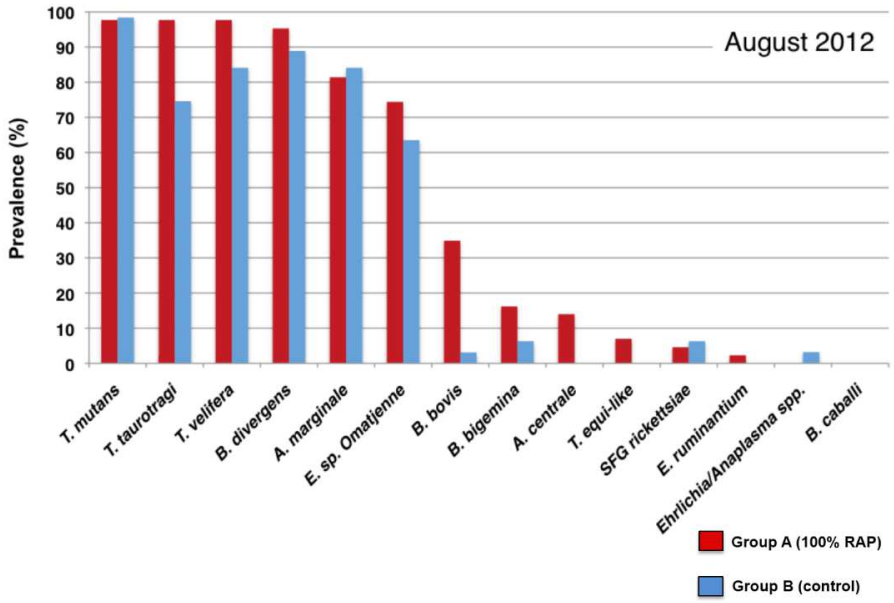
bovis, *A. centrale* and *T. equi*-like microorganisms were recorded in more variable prevalence (especially in Group A), while some other microorganisms, namely SFG rickettsiae, *Ehrlichia*/*Anaplasma* spp. and *B. caballi*, were never detected above the threshold of 10% (see Figure 4.3, a–e).



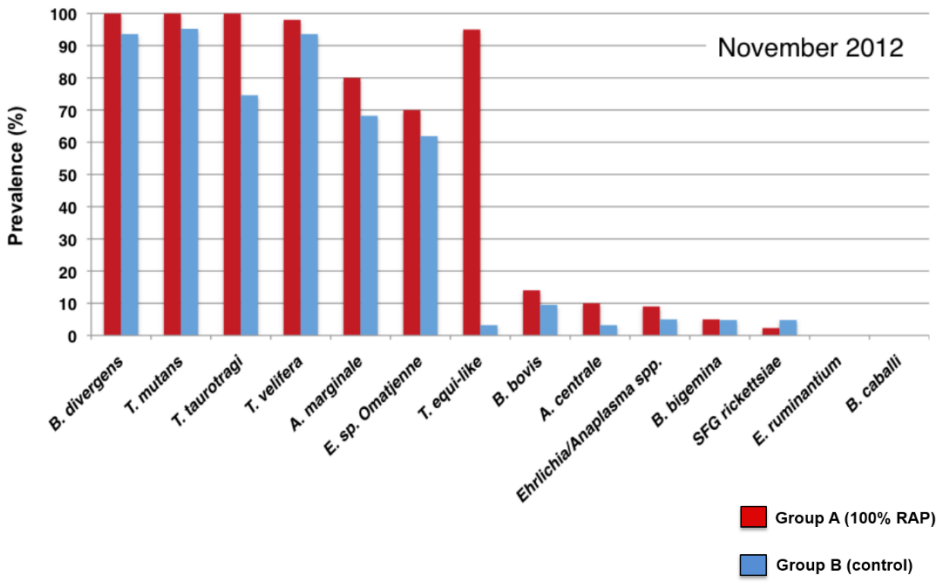
(a)



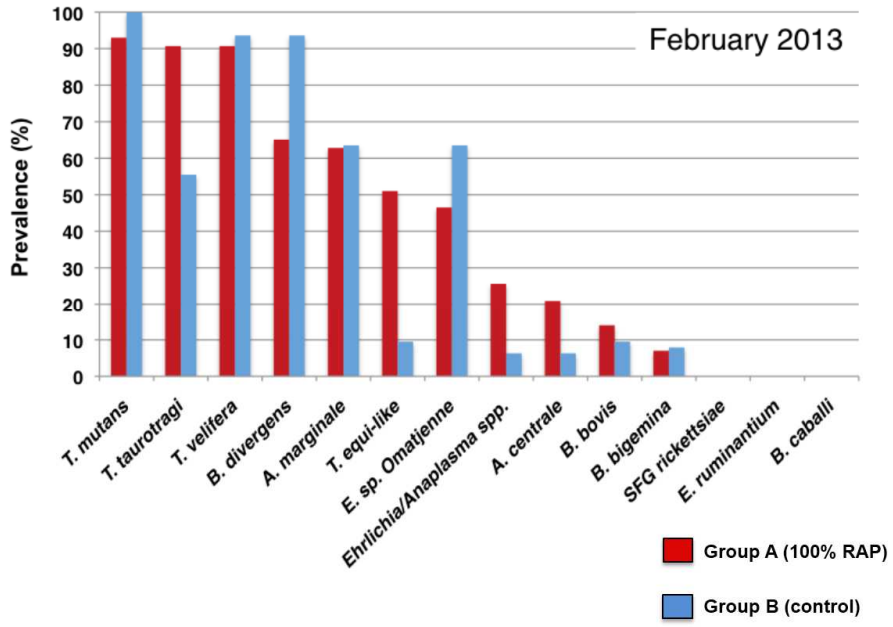
(b)


August 2012
Prevalence (%)
100
90
80
70
60
50
40
30
20
10
0
T. mutans
T. taurotragi
T. velifera
B. divergens
A. marginale
E. sp. Omatjenne
B. bovis
B. bigemina
A. centrale
T. equi-like
SFG rickettsiae
E. ruminantium
Ehrlichia/Anaplasma spp.
B. caballi
Group A (100% RAP)
Group B (control)

(c)


November 2012
Prevalence (%)
100
90
80
70
60
50
40
30
20
10
0
B. divergens
T. mutans
T. taurotragi
T. velifera
A. marginale
E. sp. Omatjenne
T. equi-like
B. bovis
A. centrale
Ehrlichia/Anaplasma spp.
B. bigemina
SFG rickettsiae
E. ruminantium
B. caballi
Group A (100% RAP)
Group B (control)

(d)



(e)

Figure 4.3 (a–e) – Prevalence (%) of each tick-borne haemoparasite detected in Group A (red histograms) and Group B (blue histograms) during the study period.

The kinetics of infections by each tick-borne microorganisms, throughout the study period, in groups A and B are elucidated in the sections below. Importantly, at baseline, no statistically significant difference between the two groups, was recorded for the following microorganisms:

- *A. marginale* (p=0.075);
- *E.* sp. Omatjenne (p=0.4);
- *B. bigemina* (p=0.6);
- *B. bovis* (p=0.1);
- *B. divergens* (p=0.5);
- *T. mutans* (p=0.5);
- *T. velifera* (p=1);
- *T. taurotragi* (p=0.4);
- *Rickettsia* spp. (p=0.6);
- *Ehrlichia/Anaplasma* spp. (p=1).

4.4.3 *Anaplasma marginale*

Prevalence of *A. marginale* ranged between 62.8% (February 2013) and 90% (April 2012) in Group A; and from 63.5% (February 2013) to 87.3% (March 2012) in Group B. Originally not significant at baseline (see Figure 4.4), the difference between the two groups became significant only in the month of April (p=0.02), with treated animals being more infected (90%) than those untreated (65%). While in the treated group the prevalence slightly decreased (reaching 81%) in August, an increase was recorded in the control group (84%), reaching very similar values in both groups (Figure 4.4). A reduction of the prevalence was then recorded in both Group A and B towards the end of the study, with very close values being registered in February 2013 (62.8% in Group A, 63.5% in Group B).

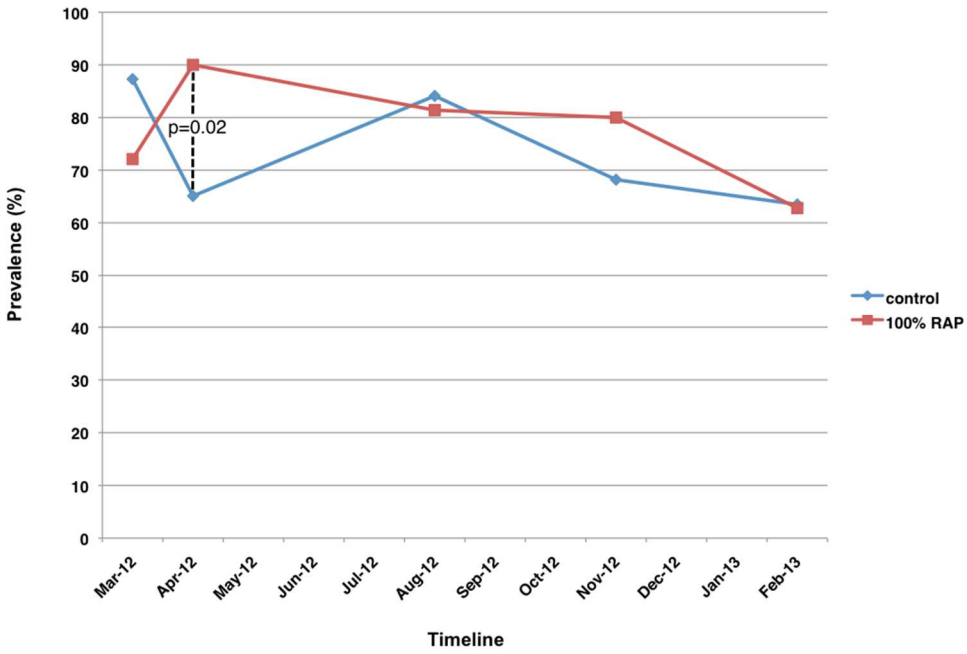


Figure 4.4 – Prevalence (%) of *A. marginale* infections in Group A (red) and B (blue) during the study period.

4.4.4 *Anaplasma centrale*

The prevalence recorded for *A. centrale* was rather different in the two groups, ranging between 10% (April and November 2012) and 20.9% (February 2013) in Group A, and 0% (August 2012) and 11.1% (April 2012) in Group B. Already at baseline, cattle in Group A (14%) were significantly more infected than those in Group B (1.6%) (p=0.02). In April 2012, the prevalence in the two groups became very close (Group A=10%, Group B=11.1%) (p=1). However, while the infection rates slightly increased in Group A (14%) in August, they dramatically decreased in Group B reaching 0%, with a difference between the two groups much more significant that in the one at baseline (p=0.004). *A. centrale*'s prevalence returned to 10% in November 2012 in Group A, while it increased to 3.2% in Group B (p=0.06). A rise of prevalence was then seen in both groups in February 2013, although the difference between them remained significant (p=0.03) (Figure 4.5).

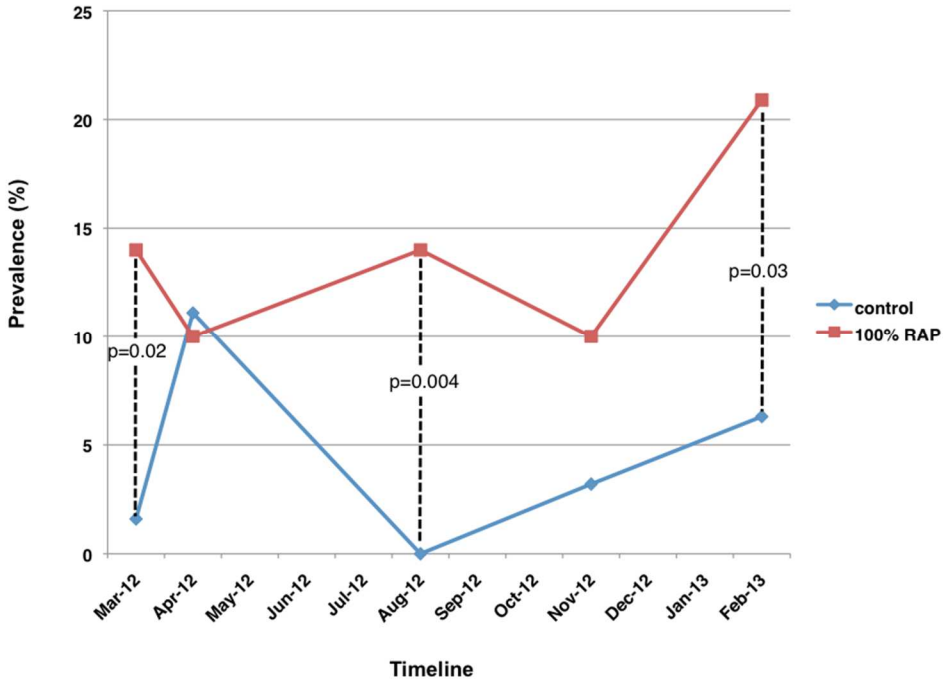


Figure 4.5 – Prevalence (%) of *A. centrale* infections in Group A (red) and B (blue) during the study period.

In Group A, the cumulative incidence (CI) of *A. centrale* infections increased rather constantly, from April 2012 to February 2013 (see Figure 4.6); whereas in Group B, a smaller number of new infections started being recorded in November 2012 and

February 2013. However, in spite of the considerably different prevalence recorded in the two groups, no statistically significant difference ($p > 0.5$) was recorded following comparison of the number of new infections in Group A and B at each sampling time.

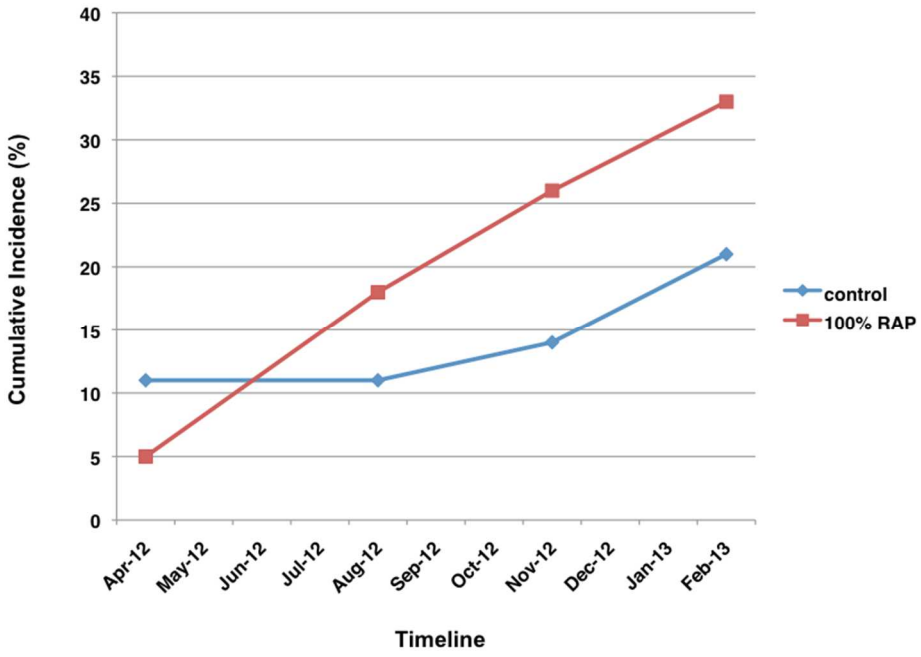


Figure 4.6 – Cumulative incidence (%) for *A. centrale* infections in Group A (red) and B (blue) during the study period.

4.4.5 *Ehrlichia* sp. Omatjenne

A rather similar trend was seen in both groups in the case of *E.* sp. Omatjenne infections. Baseline prevalence was rather similar in Group A (86%) and B (79%), followed by a rather dramatic decrease in the month of April 2012, when the prevalence in Group A (51%) was significantly higher than in Group B (30%) (see Figure 4.7). In August and November the infection kinetics followed a similar trend in both groups, increasing majorly during the wet season, and settling to a close prevalence until November (see Figure 4.7). In February 2013, the prevalence decreased to < 50% in Group A, while it remained stable to > 60% in Group B, although no statistically significant difference was recorded.

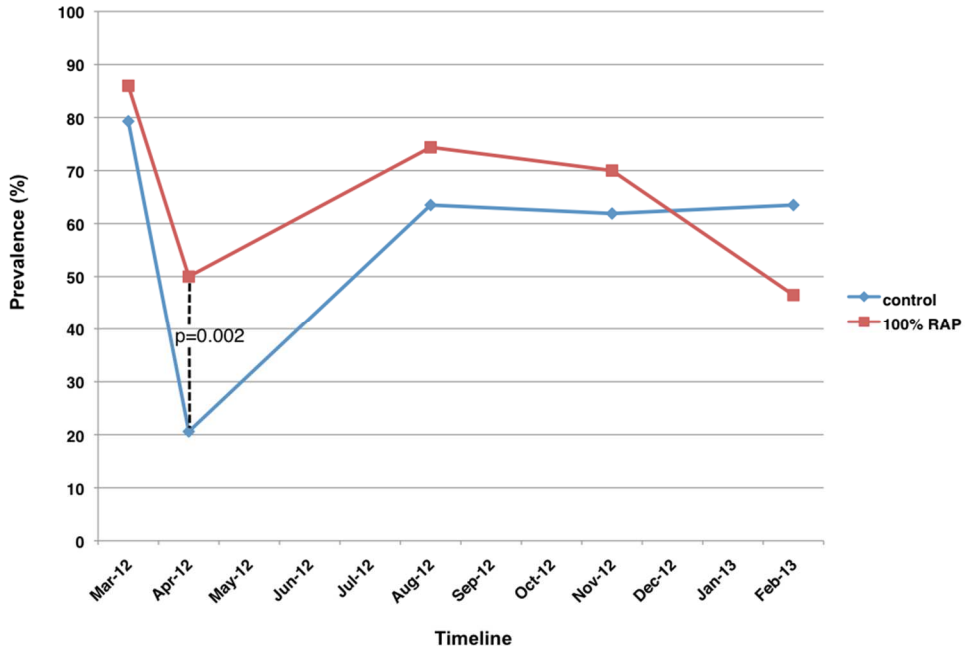


Figure 4.7 – Prevalence (%) of *E.* sp. Omatjenne infections in Group A (red) and B (blue) during the study period.

4.4.6 *Babesia bigemina* and *Babesia bovis*

At baseline, within both Group A and B, a significantly higher prevalence of *B. bovis* compared to *B. bigemina* was recorded (27.9% vs 4.6% in Group A, p=0.007; and 15.9% and 1.6% in Group B, p=0.009). In Group A, *B. bovis* was always more prevalent than *B. bigemina* during all remaining follow ups (August, November and February), and rather similarly, in Group B, *B. bigemina* was more prevalent that *B. bovis* (6.3 vs 3.1%) only at the sampling time of August 2012.

4.4.6.1 *B. bigemina*

At baseline, both groups presented a prevalence < 5%.

As expected (see also Section 4.3.4), in both Group A and B the treatment with diminazene aceturate cleared both *B. bigemina* and *B. bovis* infections, with no positive cases being recorded in the two groups in late April 2012, approximately two weeks after the second administration of the aforementioned injectable compound (see Figure 4.8 and 4.10). Later on, the wet season (August 2012) was characterised by an increase of prevalence, that was however much more pronounced in the treated (16%) than in the control group (6%), although not accompanied by a statistically significant difference (p=0.1). After the rains, in both groups the prevalence decreased to the initial value of ~5%, increasing slightly again towards February 2013 (see Figure 4.8).

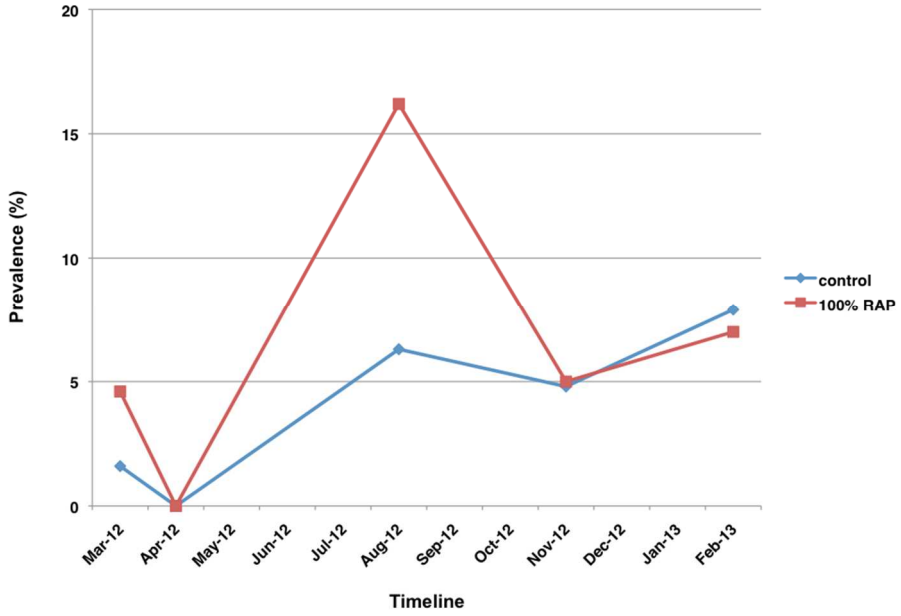


Figure 4.8 – Prevalence (%) of *B. bigemina* infections in Group A (red) and B (blue) during the study period.

In August 2012, new infections were recorded in both Group A (n=7) and B (n=4). In particular, Group A reached a CI (16%) greater than the double of that of Group B (6%) (p=0.1), while in November and February (p=0.4 and p=0.3, respectively) remained less pronouncedly higher (8 points of percentage) than that of Group B (see Figure 4.9). No statistically significant difference was found comparing the two groups' CIs throughout the study period. All of the 11 different *B. bigemina* positive cases recorded in Group A corresponded to animals older than 2 years, while 8/11 cases of Group B were found in juvenile cattle. In Group A, only one of the nine positive cases recorded after April 2012, was recorded in multiple (n=2) sampling times (i.e. August and November), and in Group B one of the 11 cases reported after April 2012 was found for three consecutive sampling times (i.e. August, November and February). Both cases were represented by adult cattle.

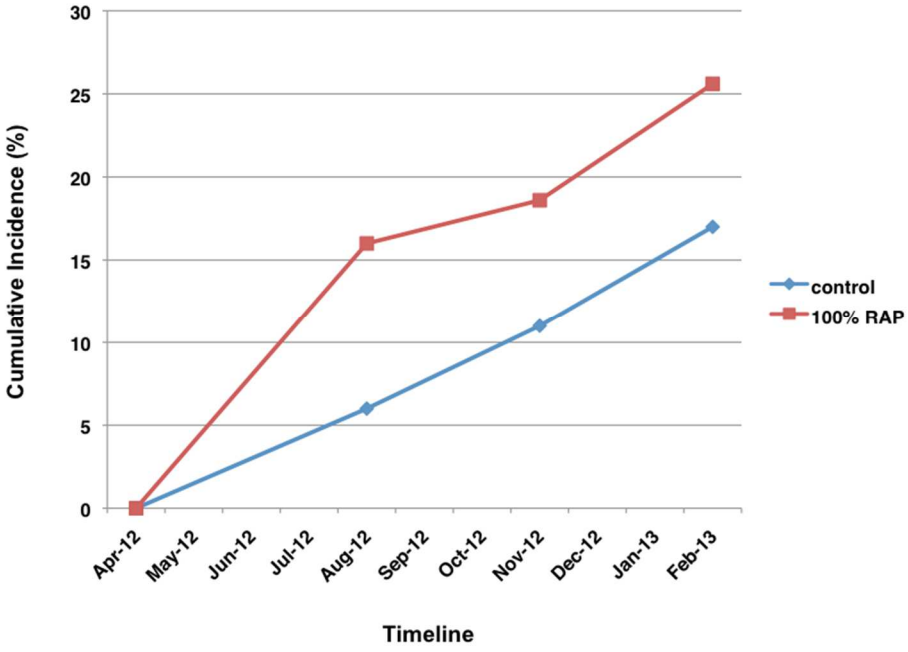


Figure 4.9 – Cumulative incidence (%) for *B. bigemina* infections in Group A (red) and B (blue) during the study period.

4.4.6.2 *B. bovis*

The infection kinetics in Group A compared to Group B likely resembled the pattern seen in the case of *B. bigemina* infection. The initial prevalence detected was considerable in both Group A (28%) and B (16%). As for *B. bigemina*, the diminazene treatment eliminated the infection in both groups (Figure 4.10). However, also in this case as with *B. bigemina*, the wet season (August 2012) was characterised by a remarkable increase of prevalence, which was significantly ($p<0.0001$) higher in the treated than in the control group (Figure 4.10). After the rains, the prevalence decreased then to below 15% in Group A, while it increased to ~10% in Group B, remaining stable in both groups until the end of the study (see Figure 4.10).

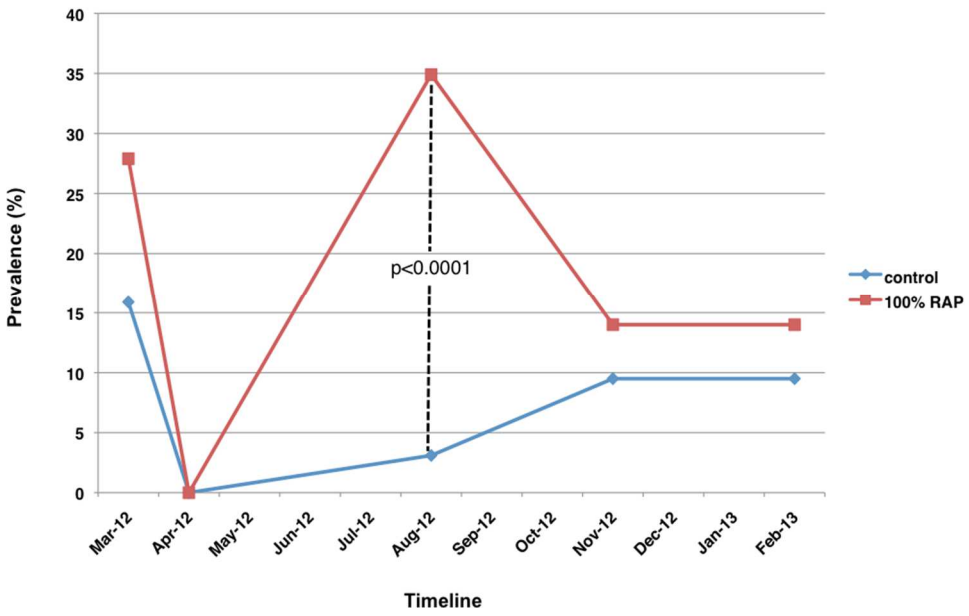


Figure 4.10 – Prevalence (%) of *B. bovis* infections in Group A (red) and B (blue) during the study period.

With regards to the CI, after the treatment with diminazene, a rather high number of new cases (n=16) were seen in Group A as opposed to Group B (n=2) ($p<0.0001$) (Figure 4.11). The difference between the CI of the two groups remained then significant in the months of November ($p<0.0001$) and February ($p=0.004$), settling on the value of 44.5% in Group A and only 17.5% in Group B.

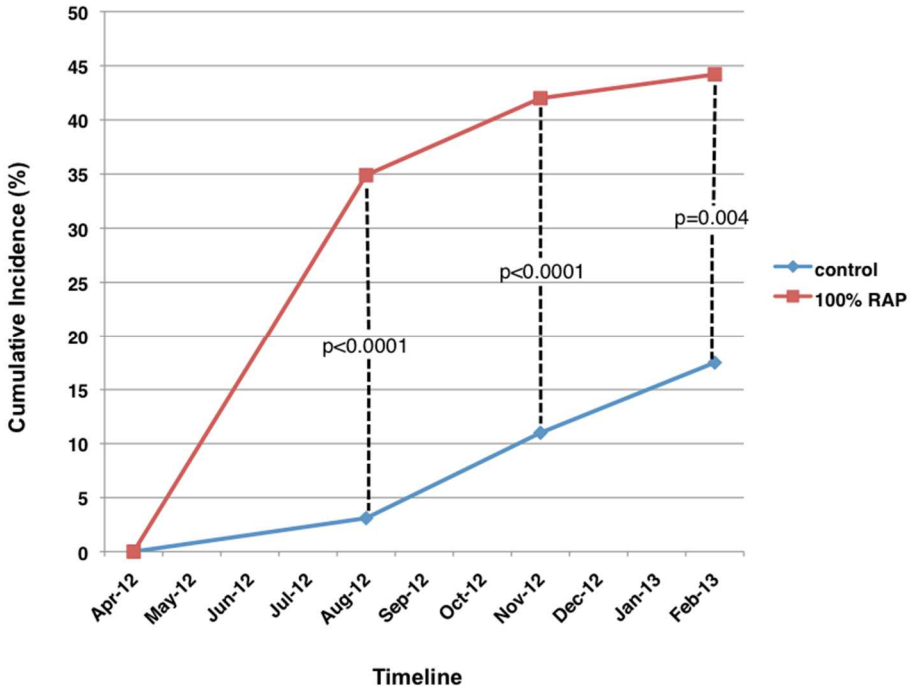


Figure 4.11 – Cumulative incidence (%) for *B. bovis* infections in Group A (red) and B (blue) during the study period.

Only one of the 26 different *B. bovis* positive cases recorded in Group A throughout the study period was aged between 6 months and 2 years, while five of the 16 positive cases recorded in Group B over the duration of the study corresponded to juvenile cattle. In Group A, four of the 19 positive cases recorded after April 2012, were recorded in multiple (n=2) sampling times (i.e. August and November; November and February; August, November and February), while in Group B only one of 11 infections reported after the diminazene treatment was reported in multiple sampling times (i.e. August, November, and February). All samples found infected at multiple sampling times were cattle of at least two years of age.

Furthermore, several cases of co-infections with both *B. bigemina* and *B. bovis* were seen throughout the study period in Group A, while they were never detected in Group B (see Table 4.5), as well as in any of the nine villages surveyed in 2008 (see Chapter 3).

Table 4.5 – *B. bigemina* and *B. bovis* positive cases in Group A and B during the study period.

	March 2012			**April 2012**			**August 2012**			**November 2012**			**February 2013**		
	B. bigemina	***B. bovis***	**Co-infections**	***B. bigemina***	***B. bovis***	**Co-infections**	***B. bigemina***	***B. bovis***	**Co-infections**	***B. bigemina***	***B. bovis***	**Co-infections**	***B. bigemina***	***B. bovis***	**Co-infections**
Group A (n=43)	2	12	1	-	-	-	7	15	3	2	6	1	3	6	2
Group B (n=63)	1	10	-	-	-	-	4	2	-	3	6	-	5	6	-

4.4.7 *Babesia divergens*

In this study, the prevalence of *B. divergens* was similar between both groups throughout the entire study period, being recorded always above 90% in group A and 85% in group B (Figure 4.12). Importantly, the diminazene treatment did not cure animals from *B. divergens*, with a prevalence of 100% and 93.6% being recorded in Group A and B respectively, in the month of April 2012 (see Figure 4.12).

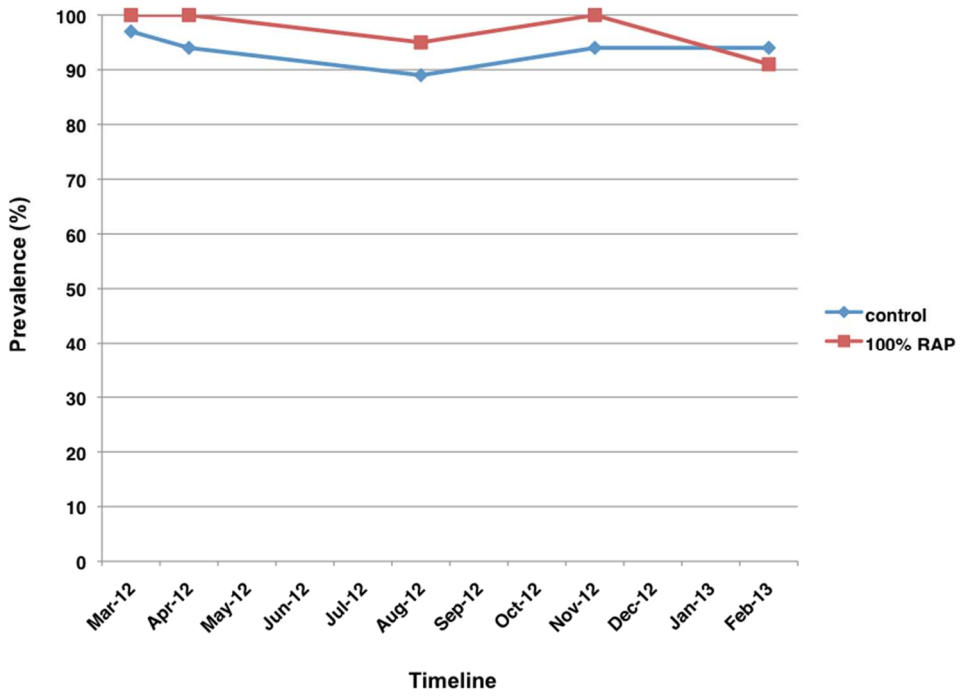


Figure 4.12 – Prevalence (%) of *B. divergens* infections in Group A (red) and B (blue) during the study period.

4.4.8 *Theileria mutans*

T. mutans prevalence was very close between both groups throughout the entire study period, being recorded always above 90% (Figure 4.13).

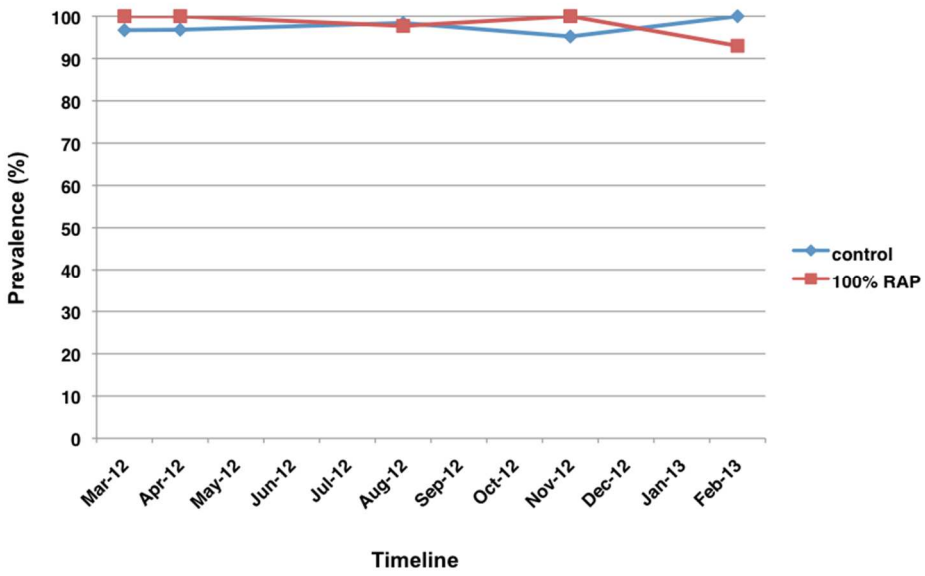


Figure 4.13 – Prevalence (%) of *T. mutans* infections in Group A (red) and B (blue) during the study period.

4.4.9 *Theileria velifera*

The trend of *T. velifera* infection was also very similar in both groups throughout the study period. Initially above 90%, in both groups, the prevalence decreased to <80% in April, increasing again throughout the wet season, and settling around 90% at the end of the study (see Figure 4.14)

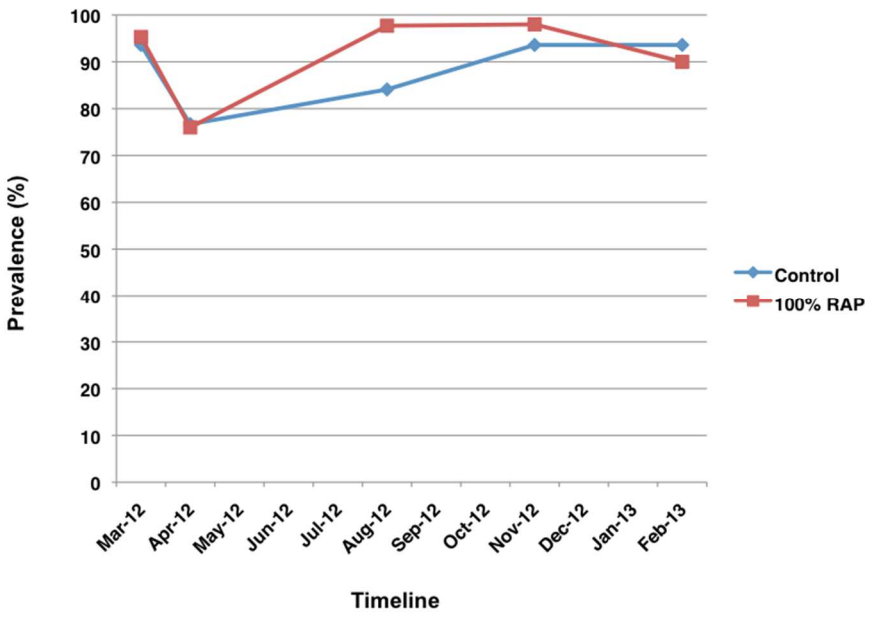


Figure 4.14 – Prevalence (%) of *T. velifera* infections in Group A (red) and B (blue) during the study period.

4.4.10 *Theileria taurotragi*

Though not significantly different (p=0.4), animals from Group A were more infected by *T. taurotragi* than those from Group B, already from the baseline, when a ~10% higher prevalence was detected in the former, compared to the latter.

Afterwards, the difference between the two groups in *T. taurotragi* infection became significant in April and in November 2012, reaching nearly the same value (~90%) in the month of February (see Figure 4.15).

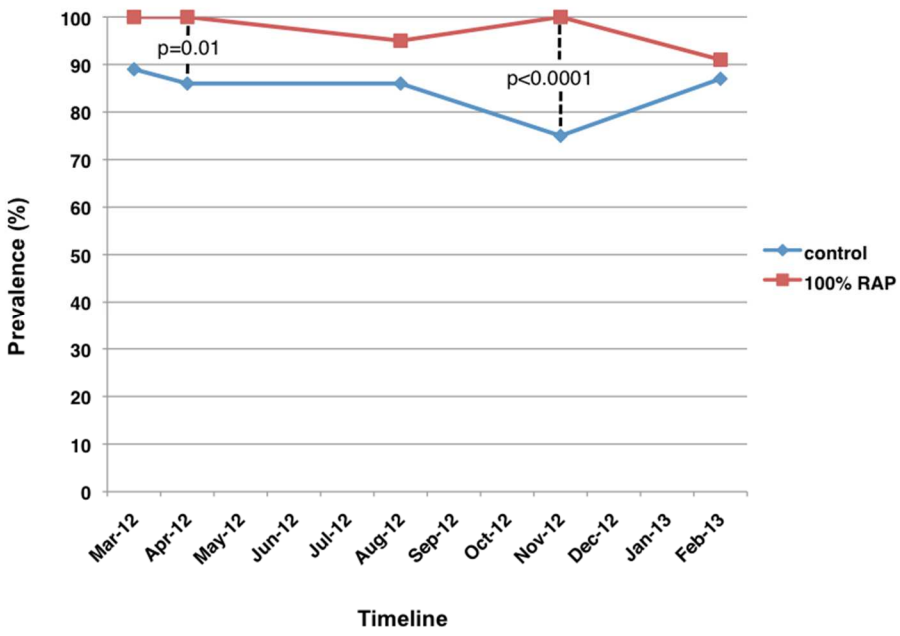


Figure 4.15 – Prevalence (%) of *T. taurotragi* infections in Group A (red) and B (blue) during the study period.

4.4.11 SFG rickettsiae

Positive cases for *Rickettsia* spp. DNA were detected in Group A and B. *Rickettsia* 'catch-all' positive samples also included positivity to 2 (i.e. *Rickettsia helvetica* + *Rickettsia* sp. (DNS14)/*raoultii* and *Rickettsia massiliae* + *R.* sp. (DNS14)/*raoultii*) to 4 (i.e. *Rickettsia conorii*, *R. helvetica*, *R. massiliae*, *R.* sp. (DNS14) /*raoultii*) species SFG rickettsiae (Figures 4.2 a–b and 4.16; Table 4.6).

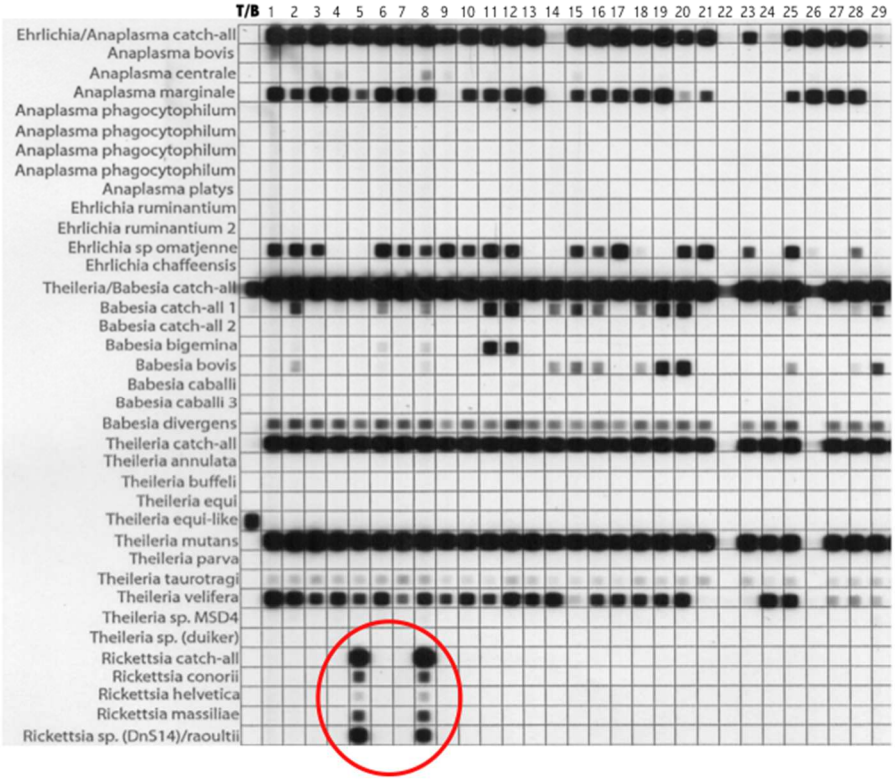


Figure 4.16 – RLB development obtained for Group A [samples 1–29] in the month of August 2012
(follow up II, showing, amongst others, blood samples positive for SFG ricketsiae (highlighted by the red circle) ['T/B': *Theileria*/*Babesia* positive control (i.e. *T. equi*)].

In both groups, cases of positivity only to individual SFG rickettsiae species-specific probes (i.e. *R. conorii*, *R. massiliae*, *Rickettsia* sp. (DNS14)/*raoultii*) that was not accompanied by a *Rickettsia* 'catch-all' signal, were also recorded. Due to the absence of a genus-specific signal before the species-specific signal, these samples were considered as doubtful and therefore were not included amongst the positive results of this study.

A rather similar trend was seen in the kinetics of SFG rickettsiae infections in both groups A and B, with no infection being detected in either treated and control animals in April, with a following increase throughout the wet season, and a further reduction during the dry season (see Figure 4.17).

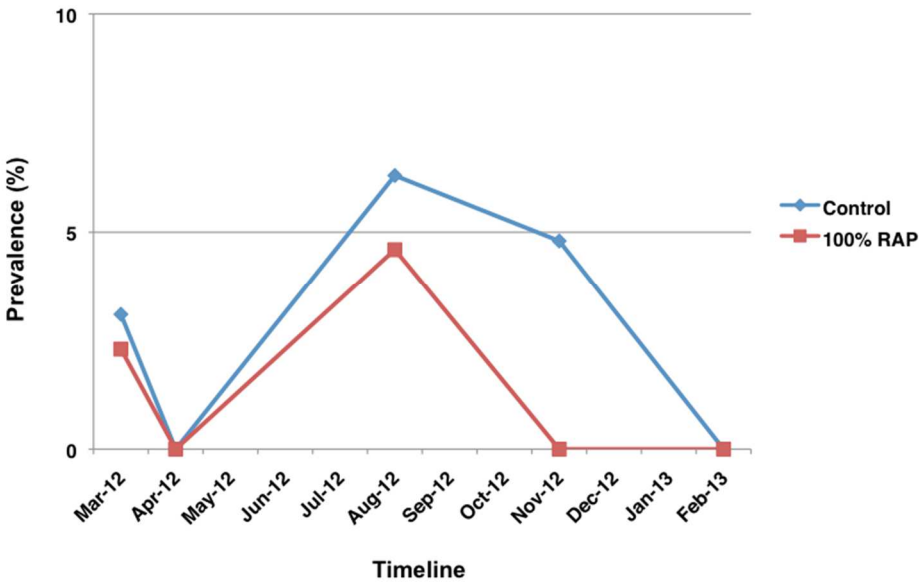


Figure 4.17 – Prevalence (%) of SFG rickettsiae infections in Group A (red) and B (blue) during the study period.

The composition of rickettsial species found amongst positive samples is summarized in Table 4.6.

Table 4.6 – SFG rickettsiae detected, simultaneously, throughout the study period in Group A and B.

	Catch-all / Species-specific positivity					**Total**
	***Rickettsia* catch-all**					
	R. helvetica	***R. massiliae***	***R. conorii***	***R. helvetica***	***R. conorii***	
	R*.sp. (DnS14) /*raoultii	***R*.sp. (DnS14) /*raoultii***	***R. massiliae***	***R. massiliae***	***R. helvetica***	
			R*. sp. (DnS14) /*raoultii	***R*. sp. (DnS14) /*raoultii***	***R. massiliae***	
					R*. sp. (DnS14) /*raoultii	
Group A (n=43)	-	-	-	-	3	**3**
Group B (n=63)	1	1	1	1	5	**9**

As inferable from Table 4.6, in Group A, all three positive cases detected throughout the study period included multiple infections by *Rickettsia helvetica, Rickettsia conorii, Rickettsia massilliae* and *Rickettsia* sp. (DnS14)/*raoultii*. A more diverse scenario was seen in Group B, where the majority of cases (n=5/9) recorded were still represented by co-infections of the four species, and one individual case of co-infections by three SFG microorganisms, and three different combinations of two species. In Group B, one same animal was found positive in August and November, resulting in the first instance positive for *R. conorii*, *R. helvetica*, *R. massiliae* and *R.* sp. (DnS14)/*raoultii*, while in the latter positive only for *R. helvetica*, *R. massiliae* and *R.* sp. (DnS14)/*raoultii*.

4.4.12 *Ehrlichia/Anaplasma* spp.

Throughout the study period, several samples turned positive only for the *Ehrlichia*/*Anaplasma* catch-all probe, with no species-specific signals being detected (Figure 4.18). Significative differences between the prevalence of the two groups were recorded in April 2012 and February 2013. In the former instance (i.e. April), the prevalence of *Ehrlichia*/*Anaplasma* spp. cases was significantly higher ($p = 0.04$) in Group B (19%) compared to Group A (5%), while in the latter (i.e. February) Group A presented with a prevalence more than four times higher than that of Group B (i.e. 26% vs 6%; $p = 0.009$; see Figure 4.18).

In Group A, two out of three positive cases of March represented the only positive animals of the month of April 2012 (see Figure 4.19). No new infections were recorded in August, while a few (n=3) new cases were recorded in the month of November 2012. A marked increase of number of new cases was then found in February 2013 (Figure 4.19).

A different trend was noted in Group B, with a number of new infections being recorded in the month of April 2012, followed by a moderate increase of positive cases in the following sampling times (Figure 4.19).

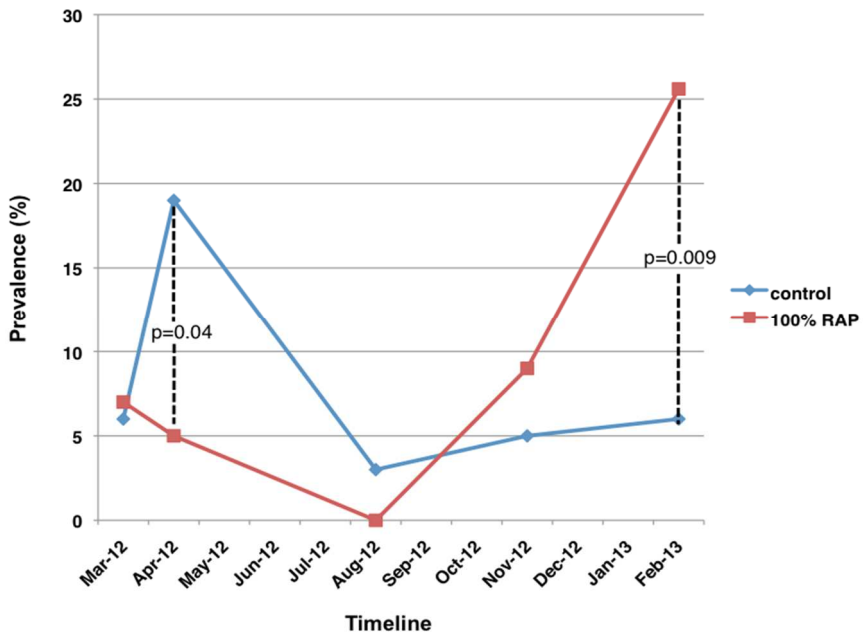


Figure 4.18 – Prevalence (%) of *Ehrlichia*/*Anaplasma* spp. infections in Group A (red) and B (blue) during the study period.

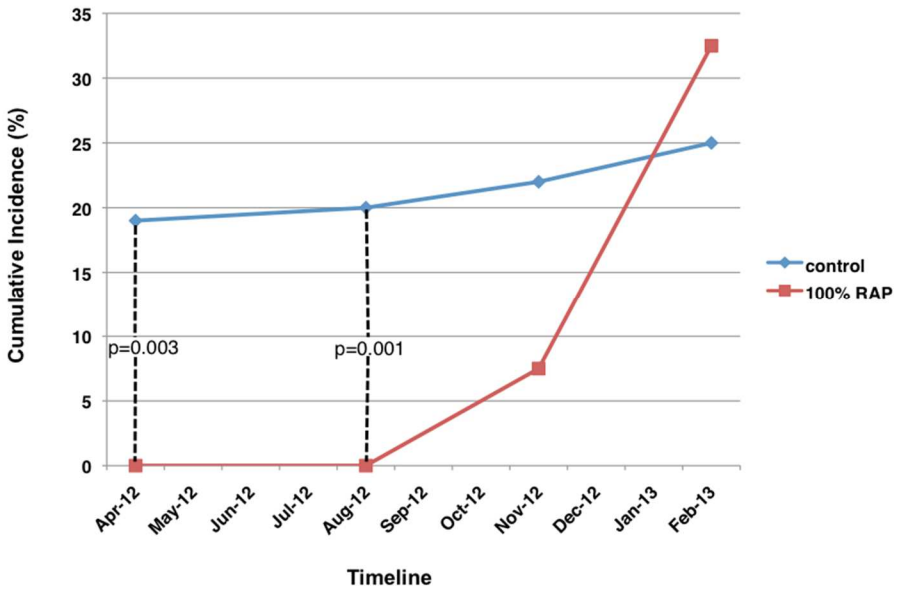


Figure 4.19 – Cumulative incidence (%) for *Ehrlichia*/*Anaplasma* spp. infections in Group A (red) and B (blue) during the study period.

4.4.13 *Babesia caballi*

B. caballi was found only in one sample of Group A and in one from Group B in March 2012 (see Figures 4.20 and 4.21).

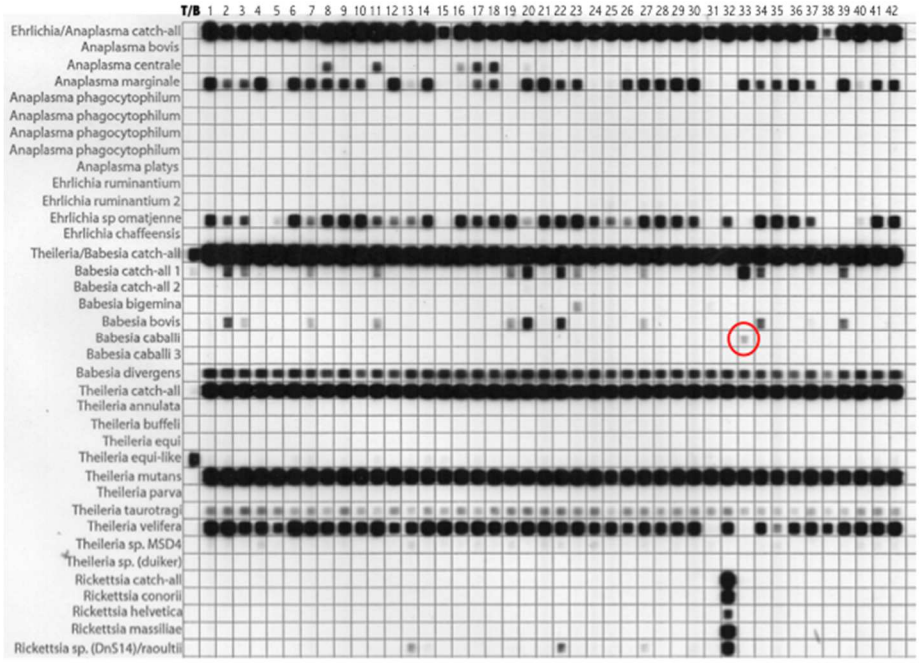


Figure 4.20 – RLB development showing a sample positive for *Babesia caballi* (red circle).

RLB development showing a sample positive for *Babesia caballi* (red circle).

[Samples from for Group A #1–42, March 2012 (baseline)].

['T/B': *Theileria*/*Babesia* positive control (i.e. *T. equi*); 'N_1': blank FTA™ paper (negative control, 1); 'N_2': MilliQ PCR water (negative control, 2)].

4.4.14 *Theileria equi*-like microorganisms

T. equi-like microorganisms DNA was detected in cattle from groups A and B at rather variable prevalences throughout the study period (Figures 4.3 a–e, and 4.21). In particular, in Group A *T. equi*-like positive samples were detected in August (7%), November (95%) and February (51%), while in Group B at baseline (28.6%), April (17.4%), November (3%) and February (9.5%) (Figure 4.3 a–e).

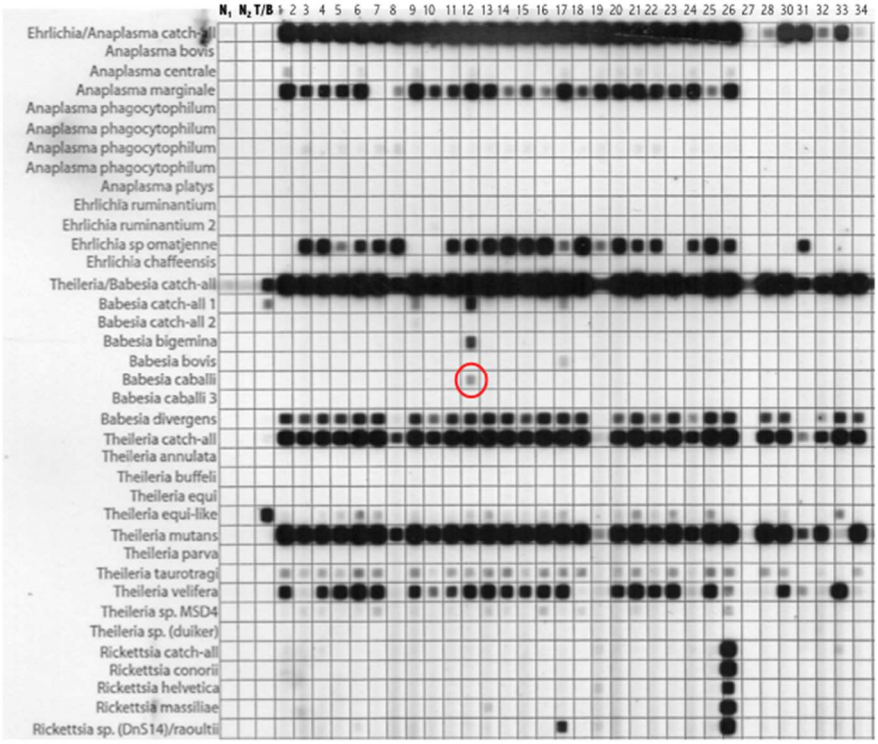


Figure 4.21 – RLB development showing a sample positive for *Babesia caballi* (red circle), as well as samples showing *Theileria equi*-like signals.
[Samples from Group B #1–34, March 2012 (baseline)].
['T/B': *Theileria*/*Babesia* positive control (i.e. *T. equi*); 'N_1': blank FTA™ paper (negative control, 1); 'N_2': MilliQ PCR water (negative control, 2)].

4.4.15 *Ehrlichia ruminantium*

E. ruminantium 16S DNA was detected in one animal from Group A (August 2012) and in one from Group B (March 2012). In both cases detection was achieved with the probe by Bekker *et al*. (2002), employed also in the survey described in Chapter 3.

4.5 Discussion

To the best of my knowledge, this is the first molecular-based longitudinal survey, monitoring the effect of a tsetse-focused control intervention on TBIs in SSA for such a length of time. In this study, the same animals in Group A (n=43) and B (n=63) were monitored over a period of 11 months.

Figure 4.22 shows the prevalence of TBIs within Hurti, where the present study took place, disclosed by the survey described in Chapter 3.

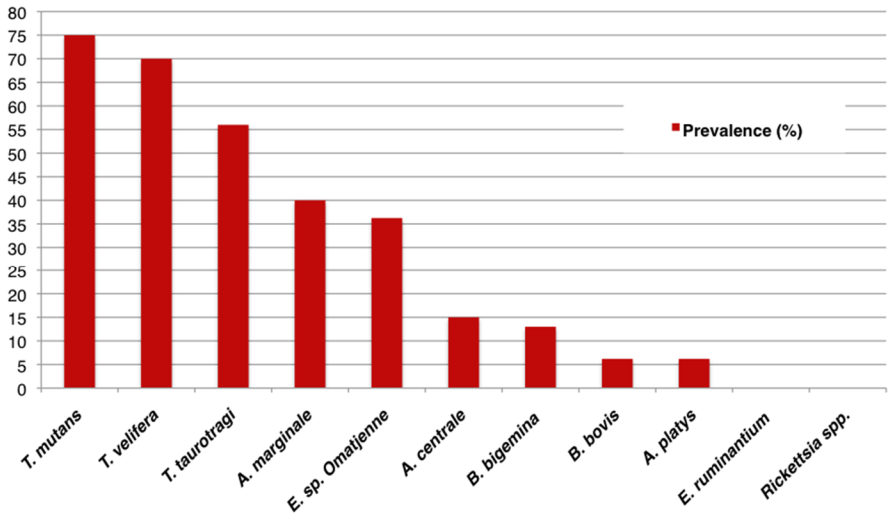


Figure 4.22 – Prevalence (%) of each tick-borne haemoparasite detected in the village of Hurti in October 2008.

As seen for the overall study area, the considerably higher prevalence of a number of microorganisms, such as *T. mutans*, *T. velifera*, *T. taurotragi*, *A. marginale* and *E.* sp. Omatjenne, compared to others (i.e. *A. centrale*, *B. bigemina*, *B. bovis* and *A. platys*), suggested the existence of a scenario of endemic (enzootic) stability for the former tick-borne haemoparasites. Conversely, the lower prevalence of the latter tick-borne microorganisms, was considered to suggest a more unstable epidemiological condition, as argued in Section 3.5. For this reason, in the present chapter, CI was calculated for these microorganisms (i.e. *A. centrale*, *B. bigemina*, *B. bovis* and *Ehrlichia*/*Anaplasma* spp.), seemingly less epidemiologically "established" in the area, to better assess the impact of this control intervention on their infection kinetics.

4.5.1 Overall infection rates

The application of a spray formulation of 0.005% deltamethrin did not influence the overall number of infected (for any TBIs) vs uninfected animals in the treated compared to control group. Both groups A and B presented very similar prevalences of infected animals (ranging from 98–100% according to the sampling times), with only two different negative animals being recorded, per group, throughout the study period (see Table 5.4).

On the whole, the overall high infection rates recorded in both groups can be explained by the fact that all animals enrolled in the study were aged ≥ 6 months (as explained in Section 4.3.3) and probably had therefore been previously exposed to the tick-borne microorganisms circulating in the area. As discussed in the sections to follow, several of these TBIs are indeed characterised by persistent post-acute infections (Eriks *et al*., 1989, 1993; Kieser *et al*., 1990).

In this study animals were screened for broader array of tick-borne microorganisms thain Chapter 3, enabling the identification of at least 25 (instead of 12) different microorganisms belonging to five different genera (i.e. *Anaplasma*, *Ehrlichia*, *Babesia*, *Theileria* and *Rickettsia* spp.; see also Section 4.3.9 and Table 4.3).

In accordance with the findings of Chapter 3 (see also Figure 4.3 a–e), the high prevalence recorded for *T. mutans*, *T. taurotragi*, *T. velifera, B. divergens*, *A. marginale*, and *E.* sp. Omatjenne, could be attributable to the presence of persistently infected 'carrier' animals, in a context of endemic stability as aforementioned. These were the haemoparasites whose prevalence was likely not affected by the pyrethroid-based treatment regimen. However, a rather more diverse scenario was seen amongst the lower prevalence tick-borne haemoparasites, with special reference to the *Babesia* species.

Results referring to single TBIs are discussed in the section below.

4.5.2 *Anaplasma marginale*

The remarkably high prevalence of *A. marginale* recorded in both groups throughout the study period suggests the lack of interference of the 0.005%

deltamethrin-based RAP on the infection kinetics of this pathogen. This finding is in line with work carried out in Zimbabwe, suggesting that the use of pyrethroids is less likely to disrupt endemic stability by *Anaplasma* spp. than other TBIs (Norval *et al*., 1984). In particular, Norval *et al.* (1984) ascertained that the frequency of dipping cattle in pyrethroid-based baths did not reduce the seroprevalence to *Anaplasma*. Similarly, work carried out in in tropical Queensland, Australia, proved that the intensity of tick control must be very high to reduce the inoculation rate of *A. marginale* in beef calves (Paull *et al*., 1980).

In particular, the high prevalence recorded in both Group A and B, seemingly reflects the existence of long-term persistent rickettsiaemia in cattle recovering from acute infections (Eriks *et al*., 1989, 1993; Kieser *et al*., 1990) and the good efficiency of the RLB assay in detecting these infections. It should be considered that transovarial transmission of *Anaplasma* does not occur in the infected (female) tick population, hence the establishment of persistent infection in the bovine host is of epidemiological relevance, in order to perpetuate the transmission of this pathogen in nature (Suarez and Noh, 2011). Once ticks become infected through the ingestion of contaminated blood from a carrier animal, *A. marginale* replicates in the lumen of the ticks' gut (i.e. Malpighi tubes), migrating afterwards to the salivary glands of adult ticks (reviewed in Aubry and Geale, 2011). Therefore *A. marginale* can be re-injected into the host by adult ticks which had been infected during previous developmental stages (e.g. larva or nymph) (Aubry and Geale, 2011). In case of massive infestation by competent adult ticks, a large boost of re-injection of *Anaplasma* may therefore take place in cattle (Aubry and Geale, 2011). Although intrastadial transmission in ticks is possible during the adult stage, when an adult tick moves from one cattle to another, this is numerically negligible for adult *Rhipicephalus* (*Boophilus*) ticks, considering that these are one-host ticks, parasitizing from larval to adult stage the same host (Walker *et al*., 2003). Though known to serve as vectors of *A. marginale* and *A. centrale* in other parts of SSA (e.g. South Africa), the role of tick species other than boophilds (e.g. *Rhipicephalus simus* Group) as vectors of *A. marginale* has not yet been ascertained for Nigeria (Leeflang and Ilemobade, 1977a,b). Therefore, ticks of the boophilid species should be considered more as amplifiers of the *A. marginale* parasitic load rather than

epidemiologically relevant vectors, considering their one-host life cycle and thus limited contribution to the spread of infection (de la Fuente *et al*., 2005). As indicated in Chapters 1 and 3, several other routes of transmission should also be considered as conditioning the epidemiology of this TBI, including mechanical transmission via biting and sucking flies (e.g. *Stomoxys* spp., *Tabanus* spp., *Chrysops* spp., *Haematobia* spp.) (Foil, 1989; Scoles *et al*., 2005; Aubry and Geale, 2011; Baldacchino *et al*., 2013). Mechanical transmission by stomoxes and tabanids can be of great epidemiological relevance, considering the capacity of sucking flies of covering distances of up to several km (e.g. 3–5 km) in a given area (Bailey *et al*., 1973; Taylor *et al*., 2010).

In the case of this study, the iatrogenic route of infection has not likely affected the prevalence detected during the study period, considering that animals were not administered any injectable drug, except for diminazene aceturate, injected using individual needles. The implication of this route of transmission, though, cannot be ruled out with reference to the establishment of the infections prior to the initiation of the study.

The prevalence of *A. marginale* infection was never below 60% in either of the two groups throughout the study period. The difference in prevalence between Group A and B was significant (p=0.02) only in the month of April, when treated animals were more infected (90%) than those untreated (65%) (see Figure 4.4). Considering that in Group B the prevalence in this case was still above 60%, this difference in prevalence between the two groups is attributed to the typical cyclic trend of infection seen in cattle persistently infected by *Anaplasma* spp., characterised by fluctuations of rickettsiaemia, within and beyond the molecular detection threshold (Kieser *et al*., 1990; see also Section 3.5.18). These fluctuations could also explain cases of animals turning ‘negative’ between two sampling times when they were found ‘positive’ (data not shown). As discussed in Section 3.5.18, the RLB method is seemingly able to detect most chronic infections in carrier animals, losing detectability in cases of parasitaemias lower than 7,000 infected red blood cells/ml of blood. Therefore, the RLB assay employed in this study cannot identify chronic infections during the low peak of the rickettsiaemic cycle, occurring

for 5–8 days every 5–6 weeks, when the parasitaemia can reach a concentration as low $10^{2.5}$ infected erythrocytes/ml of blood (Eriks *et al*., 1989).

4.5.3 *Anaplasma centrale*

The significantly higher prevalence of *A. centrale* in the treated compared to the control group at two follow ups (i.e. August and February) could be justified by the already existing difference (p=0.02) between the two groups at the baseline of the study, which may reflect inter-herds differences present within the village of Hurti. Remarkably, however, during the wet season (August 2012), this difference between the two groups became more significant (p=0.004), with the prevalence of Group A increasing (from 10% to 14%), while reaching 0% in Group B (see Figure 4.5).

In Nigeria, *A. centrale* can be transmitted by *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *annulatus* (Aubry and Geale, 2011), and possibly also by *Rh. simus* Group ticks (Potgieter and van Rensburg, 1987). As already discussed in Chapters 1 and 2, these species usually reach their greatest abundance in Nigeria in the wet season (i.e. between June and October) (Bayer and Maina, 1984). Therefore, the moderate increment recorded in the treated group, in spite of the RAP, could be a reflection of the seasonality of their tick vectors. Considering that in August 2012, as well as in February, new cases of infections kept being recorded, it is likely that this treatment regimen did not prevent the transmission of this microorganism. Otherwise, the fact that in August infections were not recorded in the control group, can be attributed to the fluctuations of parasitaemia taking place in cattle during the chronic phase of infection, with the rickettsiaemia falling below the RLB detection threshold for a few days approximately every month and half (Eriks *et al*., 1989; Suarez and Noh, 2011, see also Section 4.5.2).

The rather modest increase of CI recorded in Group A, in presence of a rather steady prevalence, at each follow up sampling, including August 2012 (see Figure 4.6), and in the light of the abundant (n=62/63) adult population in this group, suggests the existence of persistent post-acute chronic infection also in the case of *A. centrale* infections.

In the case of Group B, instead, the modest CI is essentially attributable to the overall small number of positive cases recorded. In this group, six out of the 14 (43%) total *A. centrale* positive cases registered throughout the study were represented by juvenile cattle between 6 and 18 months of age, thus very likely representing first cases of exposure after the vanishing of the passive maternal first and innate protection after (Kocan, 1995).

In Section 3.5, *A. centrale* was considered, possibly together with *B. bovis*, as potentially epidemiologically 'unstable' in the Nigerian Plateau, in the light of the overall prevalence of 6.3% (95% CI: 4.2–8.3%). In the village of Hurti alone, however, the prevalence of this microorganism reached 15% (see also Figure 4.22). In the present study, screening animals for the period of nearly a year enabled a clearer view to be obtained in this respect. On the whole, the prevalence of *A. centrale* was always below 25% in Group A (mean = 14.6%) and 15% in Group B (mean = 4.4%), being considerably lower than the respective mean prevalence of 76.7% and 73.6% recorded for *A. marginale*. This considerable difference of prevalence could be possibly attributed, to a lower tick transmission efficiency of *A. centrale* compared to *A. marginale*, not only infecting fewer ticks than the latter, but also replicating to a lesser extent in the tick midgut and salivary glands (Ueti *et al*., 2009). This would count especially for the juvenile animals of this study. For the adults, however, a parasitaemia beyond the threshold of detectability during the post-acute phase of infection (Eriks *et al*., 1989) should also be considered. This is in line with the findings of Chapter 3**,** as well as with a previous survey (Kuttler, 1972), disclosing significantly lower rickettsieamia in adult cattle infected by *A. centrale* compared to those inoculated with *A. marginale*.

4.5.4 *Ehrlichia* sp. Omatjenne

The study described in Chapter 3 ascertained, for the first time, the presence of *E.* sp. Omatjenne in Nigeria. This tick-borne microorganism, of yet not fully clarified pathogenicity to cattle (Du Plessis, 1990; Allsopp *et al*., 1997), was found at a prevalence of 36% in the village of Hurti in 2008 (see Figure 4.22). The present survey disclosed an even higher prevalence of this TBI in this village at the baseline

sampling of March 2012, when rather close prevalences were recorded in Groups A and B (i.e. 86% and 79.3% respectively, see Figures 4.3a and 4.7). On the whole, a similar trend of the infection kinetics was recorded in both groups throughout the study period. The decline of the prevalence in both Group A and B, registered in April, followed by the increase of August 2012, can be attributed to the seasonality of the tick species most likely implicated in the transmission of this microorganism in Nigeria, namely *H. truncatum* (Du Plessis, 1990).

Despite the fact that the difference of prevalence of the two groups was statistically significant (p=0.002) in April, when it reached 50% in Group A and 20.6% in Group B, it is still not believed to be induced by the treatment, considering that both groups experienced a decline in the prevalence at that time. Altogether, the *E.* sp. Omatjenne prevalence was never lower than 45% in Group A (mean: 65.3%) and never lower than 20% in Group B (mean: 57.8%), highlighting, consistently with the previous findings of Chapter 3, the endemic establishment of this tick-borne microorganism in the study area.

4.5.5 *Babesia bigemina* and *Babesia bovis*

Following the survey described in Chapter 3, this study further confirms the occurrence of *B. bovis* in the study area. This is of relevance considering the great veterinary importance of this piroplasm species (Bock *et al.*, 2004), and the fact that farmers from the area often report haematuria in their cattle (Santirso[14], personal communication).

In addition to the 2008 survey (see Chapter 3), this study provides more detailed information on the kinetics of both *B. bigemina* and *B. bovis* infections throughout the year, in the Plateau. In Nigeria, the species known as the vector of *B. bigemina* is *Rh.* (*Bo.*) *decoloratus*, the most widespread tick over the Plateau (Chapter 2 and Lorusso *et al.*, 2013a). Conversely, the vector competence for *B.*

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bovis has not yet been fully clarified. As discussed in Section 4.5.8, other boophilids identified in the study area that can potentially serve as vectors of *B. bovis* include *Rh.* (*Bo.*) *annulatus* (Bock *et al.*, 2004) and *Rh.* (*Bo.*) *geigyi* (Akinboade and Dipeolu, 1981).

The significantly higher baseline prevalence of *B. bovis* compared to *B. bigemina* recorded (p=0.007 in Group A; p=0.009, in Group B), represents a rather striking finding, considering that in the survey described in Chapter 3, *B. bigemina*'s prevalence (7.9%) predominated over that of *B. bovis* (1.9%). Comparing the two groups for the same infections, no statistically significant difference was recorded with regards to both *B. bigemina* (p=0.6) and *B. bovis* (p=0.1) infections at baseline.

After baseline, all animals tested negative for *B. bigemina* and *B. bovis* infection in Group A and B, suggesting the curative efficacy of the treatment with diminazene aceturate at two weeks intervals. At present, diminazene aceturate is the most used anti-trypanosomal agent (Peregrine, 1994) and, together with imidocarb diproprionate, it is one of the two most indicated drugs against babesiosis (Mosqueda *et al*., 2012). Assuming all animals cleared the infection after the double diminazene treatment, from April 2012 onward, for both groups, the denominator of the formula used for the calculation of the CI, for *B. bigemina* and *B. bovis* infections, included the entire herd (n=43 for Group A, and 63 for Group B).

In August, however, positive cases for both *B. bigemina* and *B. bovis* were recorded in Group A and B. Diminazene's efficacy against these two babesias is indeed known to last for four (*B. bigemina*) and two (*B. bovis*) weeks respectively (de Vos, 1979). This, together with the high challenge by the *Babesia*-vectoring boophilid ticks during the wet season (i.e. June to October) (Bayer and Maina, 1984; see also Chapter 2), could have likely caused the occurrence of new infections. In particular, the prevalence recorded for *B. bigemina* was considerably higher in Group A (16%) than in Group B (6%) (Figure 4.8), and the prevalence of *B. bovis* was significantly higher ($p<0.0001$) in Group A (34.9%) than in Group B (3.1%) (Figure 4.10). Moreover, a remarkable difference was seen in the CI with regards to both *B. bigemina*, higher in Group A (16%) than in Group B (6%), and *B. bovis*, significantly higher ($p<0.0001$) in Group A (37%) than in Group B (3%). Furthermore, for *B. bovis* infection, from this sampling time until the end of the study the CI remained

significantly higher in Group A than Group B (see also Figure 4.11), indicating that a number of new cases continued being recorded in the treated, rather than in control, group. In particular, in Group A the final CI for *B. bigemina* was 25.6% and for *B. bovis* was 44.2%. In Group B, the final CIs were 16% for *B. bigemina* and 11% for *B. bovis* infection, probably reflecting the higher inoculation rate of the former pathogen, in an area where both piroplasms are present (Mahoney and Mirre, 1971).

As already discussed in Chapter 1 and 3, the dynamics of transmission of *Babesia* parasites depend on several variables intrinsic to the three main elements conditioning the epidemiology of these piroplasms: their tick vectors, the babesial parasites themselves and the bovine host (see Table 4.7).

Table 4.7 – Variables involved in *Babesia* spp. transmission and epidemiological element to which they relate (Adapted from Smith, 1984).

Stage	Variable
Tick vector	**a.** Climatic variations
	b. Chemical control methods
	c. Innate and acquired resistance of cattle to tick feeding
	d. Density-dependent factors affecting host-finding
	e. Pasture rotation –interval
Babesia parasite	**a.** Use of chemotherapeutic agents
	b. Level of herd immunity
	c. Climatic variations affecting the life cycle in the vector
	d. Species of the parasite
	e. Breed susceptibility of cattle
	f. Infection rate in tick vector population
	g. Infection rate in bovine host population
Bovine host	**a.** Rate of entries into and removals from the herd
	b. Age structure of the herd

As already illustrated in Chapter 2, August falls within the period of the rains in Central Nigeria (Odumodu, 1983), when most tick species find the most favorable conditions for their development (Bayer and Maina, 1984). Therefore, the increase of prevalence of both *B. bigemina* and *B. bovis* recorded in August in both Group A and

B, is likely related to the higher abundance of the tick vectors of this piroplasms at this time of the year, when the egg hatching in the environment is intensified due to warmer and more humid climate (Spickett and Heyne, 1990). Boophilids are one-host ticks, developing from larvae to adults on the same host (Walker *et al*., 2003; Figure 4.23).

Figure 4.23 – Schematic representation of *Rhipicephalus* (*Boophilus*) spp. life cycle.

Their life cycle is usually rapid, and their development from larva to adult may be completed in only three weeks in the presence of the favorable conditions of the wet season. In the latter circumstance, it may also take only two months for egg laying, hatching and larval development to be completed (Walker *et al*., 2003).

As already clarified in Chapter 1, among the other TBIs, the epidemiology of *Babesia* spp. stands out for the occurrence of transovarial transmission, from an engorged female to the offspring. (Bock *et al*., 2004). Adult female ticks usually become infected in the last 24 hours of engorgement, while feeding on a parasitaemic host (Cafrune *et al*., 1995). Thereafter, they soon drop off the host to lay eggs in the environment (Walker *et al*., 2003). Alone, in fact, they are not able to transmit the infections to a susceptible host, as the infective form of *Babesia* only develops through a passage in the tick egg, first, and the larvae, after (reviewed in Bock *et al*.,

2004). Once in the midgut of the adult tick, the babesial gamonts (gametocytes), still in the erythrocytes of the engorged vertebrate host's blood undergo a multiplication, forming single-nucleated (haploid) ray bodies (Mackenstedt *et al*., 1995). After emerging from the erythrocytes, the gamonts fuse in pairs ('syngamy') forming a zygote (Bock *et al.*, 2004). The zygote infects then the tick gut cells, where it developing into a kinete ('schizogony') that gains the haemolymph space infecting other tissues too, including the oocytes. Here a secondary schizogony takes place (Bock *et al.*, 2004). Therefore, once in the tick larvae, via the egg hatching, the development of the babesias is not yet completed. In the larvae a further process known as 'sporogony' occurs, with the formation of 'sporozoites', the infective stages of babesias (Mackenstedt *et al*., 1995). Their development usually begins, once the *Boophilus* larvae have attached to the vertebrate host (Bock *et al*., 2004). In the case of *B. bigemina*, some further development needs to take place in the larvae while feeding, with infective sporozoites appearing in about 8–10 days from the attachment, ready to be transmitted to cattle, thus occurring only in the nymphal and adult stages of boophilid ticks (Hoyte, 1965; Potgieter and Els, 1977). With regards to *B. bovis*-infected larvae, sporozoites are transmitted to the cattle host after a maturation period of at least 2 or 3 days post-attachment (Riek, 1966; Mahoney and Mirre 1979). Therefore, the pre–patent period for *B. bovis* is usually of 6–12 days with peak parasitaemia being reached about 3–5 days after (Callow, 1979).

Because of their 'one host' life cycle, ticks of the sub-genus *Boophilus* are amongst the first to be affected by acaricides, first in terms of sensitivity (Davey and Ahrens, 1984), and then also resistance in cases of persistent use of the same molecules (Lovis *et al.*, 2013). At present, however, development of acaricide resistance is an event not likely to occur amongst the ticks of the Nigerian Plateau, considering the lack of chemical control employed in the whole area (see also Chapter 2). Moreover, in this study, several other expedients, such as concentration of active ingredient, the protocol followed and the duration of the trial, helped preventing this risk.

Given the limited extension of its application, the 0.005% deltamethrin employed in this study likely displayed limited 'residual (or persistent) activity', especially during the wet season, when the lower quarters of cattle become usually

wet by the contact with the humid vegetation (Stachurski and Lancelot, 2006). Therefore, this study's RAP essentially caused a short-term detachment of moribund (i.e. 'knocked down') ticks normally localized on the body areas targeted by the intervention. After treatment, the only developmental stages of boophilid ticks that could have parasitized *ex novo* the animals were indeed larvae (Walker *et al*., 2003; see Figure 4.23). In this way, the application of the 0.005% deltamethrin could have enhanced a quicker turnover of boophilids in animals of Group A compared to Group B, especially over the period of the rains. Considering that larvae may transmit *B. bovis*, while nymphs *B. bigemina*, it is possible that the application of the RAP might have indirectly favored the transmission of babesias to cattle at this time of the year. In this study, the 0.005% deltamethrin was applied to animals of Group A with a frequency of one month. According to the aforementioned, the interval of a month between applications represents a sufficiently long time span for a new generation of hatched larvae to parasitize cattle and re-inoculate them with infective *Babesia* sporozoites. In this respect, it should be also noted that, unlike *B. bigemina*, heat stimulation of larval tick prior to attachment (e.g. 37ºC for 3 days and 30ºC for 8 days) enables the immediate transmission of *B. bovis* from infected 'just attached' larvae to cattle (Dalgliesh and Stewart, 1982). This may also explain why the difference between the treated and control group was more pronounced with regards to *B. bovis* than *B. bigemina* infection.

In this respect, it is also important to underline that female ticks feeding on cattle with persistent infections though during phases of low parasitaemia are still able to acquire the infection, successfully passing the babesial kinetes to their offspring (Howell *et al*., 2007). Therefore, infective ticks certainly kept developing in the environment surrounding the treated herd in Hurti, in spite of the treatment with diminazene (Waldron and Jorgensen, 1999), first, and the RAP, after, with the latter having a too low concentration of deltamethrin to have an impact on environmental control (Vale *et al.*, 1999). Moreover, it should be noted that *B. bigemina* can be transmitted through several generations of boophilid ticks not feeding on parasitaemic hosts while *B. bovis* can be transmitted between two generations of ticks without adult females feeding on infected cattle (Potgieter and Els, 1977; Callow, 1979).

As opposed to Group A, after the diminazene treatment, animals from Group B were more gradually re-challenged by infected boophilid larvae, as the tick burden on these animals was not altered by any treatment. This may explain the less steep CI recorded in this group (Figures 4.9 and 4.11).

The hypothesis of a more gradual re-infestation by boophilid ticks of untreated animals compared to those sprayed, is supported by a number of studies ascertaining the existence of density dependent mortality factors regulating tick populations on genetically resistant (i.e. *B. indicus*) cattle (Sutherst *et al*., 1973). With regards to one-host ticks, to date these phenomena have been investigated for *Rh*. (*Bo*.) *microplus* in indicus cattle from Australia (Wharton *et al*., 1973). These studies showed the occurrence of intra-specific competition between parasitic ticks of the same developmental stage, regulating the tick burden on the host (Sutherst *et al*., 1973). As increasing rates of infestation may enhance the expression of resistance on the host (e.g. grooming, flickering of the skin, etc.), causing higher parasite killing (Anderson, 1976), certain ticks have evolved mechanisms of intra-specific competition enabling them to keep their burden below a certain threshold (Randolph, 1979). Such intrinsic 'self-regulatory' mechanisms within the tick population could be interpreted as an evolutionary adaptation of ticks to their host resistance (Sutherst *et al*., 1973). This generates a balanced relationship between the parasitized host and successfully feeding ticks (Randolph, 1979) in absence of any interfering control practice. Supposedly, density dependent regulatory factors may play a major role in the parasitism of one-host compared to two- and three-host ticks, considering the tighter relation evolutionarily established between boophilids and their cattle host.

With special reference to *B. bovis* infection, after an acute phase lasting for 1 to 2 weeks, during which parasitaemia can peak to a value of 10^{-3}% (Calder *et al*., 1996), an early post-acute phase usually takes place, during which the host immune response seems capable of suppressing the parasitaemia to levels below the threshold of PCR-based detectability (i.e. $<10^{-7}$%) (Calder *et al*., 1996). In these regards, as discussed in Section 3.5.2, it should be considered that RLB sensitivity for detection of *Babesia* spp. was ascertained to be of 10^{-6}% for *B. bovis* (Gubbels *et al*., 1999). This might help explain the dramatic reduction in *B. bovis* prevalence recorded in

Group A in November 2012 (i.e. 14%), compared to August 2012 (i.e. 35%) (Calder *et al*., 1996). In particular, fluctuations in post-acute infections in 'carrier' animals seem to follow a bimodal trend, with an early period (i.e. less than 150 days post-infection, dpi) followed by a late stage period (i.e. from 150 dpi onwards) (Calder *et al*., 1996). Interestingly, seemingly, in the first 150 dpi, *B. bovis* parasitaemia levels drop beyond the limit of PCR detectability for a longer period (i.e. up to 30 days) than during late infections, when these periods of 'undetectability' are shorter (i.e. less than 14 days) (Calder *et al*., 1996). This is of great interest, considering that, in Group A of the present study, only one of the 12 newly positive animals detected in the month of August, when a peak of both prevalence and CI was recorded, was found positive again at a further sampling time (i.e. February 2013).

Not surprisingly, the prevalence of both *B. bigemina* and *B. bovis* decreased (below 10% and 15%, respectively) in both groups in the period between November and February (see Figures 4.8 and 4.10). This corresponds indeed to the onset of the dry season, when the RH in the Plateau State considerably decreases with rather dramatic temperature shifts between hot days and cold nights (Odumodu, 1983). These conditions, altogether, are known for being less favorable to tick development (Bayer and Maina, 1984) and also to the development of babesias within the ticks (Simuunza *et al*., 2011). Therefore, not only the number of boophlid ticks are lower in the dry season, but also their transmission potential of babesias is reduced, as already demonstrated for *B. bigemina* whose development in *Rh*. (*Bo*.) *microplus* is inhibited (if not completely eliminated) by persistent high temperature above 37°C (Kocan, 1995).

Moreover, the present study confuted the hypothesis raised in Chapter 3 on the possible occurrence of competition between *B. bigemina* and *B. bovis*. In this study indeed, several cases of associations of both species were recorded (see Table 4.5), confirming the possible coexistence of the two species in the blood stream of the same host already observed in previous serology- and cytology-based (Viseras *et al*., 1999) and molecular studies (Martins *et al*., 2008; Ibrahim *et al*., 2013). These results highlight the usefulness of RLB in identifying co-infections – often occurring in such contexts – otherwise unlikely disclosed by other cytological and individual species-specific PCRs.

The relevant presence of *B. bovis* in this area highlights the need to elucidate its epidemiology in Nigeria, identifying the tick species involved in its transmission.

4.5.6 *Babesia divergens* – a highly prevalent zoonotic agent

This study confirmed the presence of *B. divergens* in cattle in the Nigerian Plateau. This microorganism's DNA was very recently retrieved, by means of PCR-based RLB targeting the 18S rDNA, in 1/153 (0.6%) *A. variegatum* ticks collected from cattle in the southern part of the Plateau State (Ogo *et al*., 2012), consistent with previous experiments detecting the presence of *Babesia*-like kinetes in the haemolymph of the same tick species (Kamani *et al*., 2011). Importantly, the present study provides the first report of the presence of this piroplasm in cattle in SSA. *B. divergens* is indeed the main and most widespread causative agent of bovine babesiosis in Europe, where its distribution approximately coincides with that of its vector *Ixodes ricinus* (Gray *et al*., 2010). Moreover, this microorganism is also implicated in cases of human infections (Zintl *et al*., 2003). Furthermore, this pathogen was also isolated from taurine (*Bos taurus*) cattle in Tunisia (Bouattour and Darghouth, 1996) within the geographical niche of *I. ricinus* (Bouattour, 1987), and it is therefore expected to be found also in Morocco (Bailly-Choumara *et al*., 1974) and Algeria (Sergent and Poncet, 1937), where the 'deer tick' is known to occur. Therefore, the finding of *B. divergens* DNA in bovine blood in Nigeria is of public health relevance for both the zoonotic importance of the agent (Zintl *et al*., 2003) and the capacity of *A. variegatum* to accidentally feed on humans (Mediannikov *et al*., 2010). The considerably high prevalence recorded for this piroplasm throughout the entire duration of the survey can be attributed to a combination of a) the continuous challenge by its possible tick vector, *A. variegatum*, documented to be well represented in the Plateau in Chapter 2; and b) the presence of a persistent parasitaemia in carrier animals recovered from active infections (Devos and Geysen, 2004). The detection of the causative agent of human (in addition to bovine) babesiosis adds to a number of other zoonotic agents (i.e. SFG rickettsiae) detected in the Fulani cattle on the Plateau. Future endeavours aiming to further ascertain the

tick vector of this apparently highly prevalent zoonotic piroplasm in Central Nigeria would be desirable.

Interestingly, the presence of *B. divergens* also at the sampling time of April 2012 (see Figure 4.12) highlights the inefficiency of the diminazene treatment against this piroplasm. Small babesias, such as *B. divergens*, have proven to be more difficult to treat than large *Babesia* spp. (Kuttler *et al*., 1981).

4.5.7 *Babesia caballi* – an equine pathogen in cattle

In this study the presence of *B. caballi* DNA was detected in two cattle, one from each group respectively (Figures 4.20 and 4.21).

To the best of my knowledge, this is the first evidence of the presence of this equine pathogen in cattle in SSA. In very recent times, DNA of *B. caballi* was retrieved in *Haemaphysalis longicornis* ticks collected from cattle in Korea (Kang *et al*., 2013) amplifying a 403 bp fragment of the V4 region of the 18S rRNA gene (Battsetseg *et al*., 2001). In SSA, *B. caballi* is known to be transmitted by *Hyalomma truncatum* and *Rhipicephalus evertsi evertsi* (reviewed in Walker *et al*., 2003). Of the two tick species, the former was ascertained to be prevalent in the Plateau State (see Chapter 2 and Lorusso *et al*., 2013a). Nonetheless, this microorganism's DNA was also found in 10/504 (2%) *A. variegatum* ticks collected from cattle in the Republic of Guinea, screened by RLB targeting the same 18S rRNA gene fragment used in the present study (Tomassone *et al*., 2005). Therefore, the presence of this protozoa in cattle in the Nigerian Plateau, may be likely attributed to the abundance of its confirmed (i.e. *H. truncatum*) and potentially additional (i.e. *A. variegatum*) vector in this study area.

The pathogenicity of *B. caballi* in cattle, known for causing equine piroplasmosis (Brüning A, 1996), is currently unknown. In this part of Nigeria, equids are not usually reared within the Fulani livestock keeping system. However, horses (*Equus ferus caballus*) and donkeys (*Equus africanus asinus*) are common in the southern part of the country (Bourn *et al*., 1992). Hence, would this molecular finding be confirmed on a more extended geographical area, its epidemiological relevance would become considerable, considering the role that cattle may

potentially play as a 'reservoir' of this microorganism, without displaying any clinically apparent condition. Similarly, the epidemiological importance of this finding may be even greater in the event of imports or simple transit of equine species (e.g. donkeys) through the Nigerian Plateau.

4.5.8 *Theileria mutans* and *Theileria velifera*

T. mutans and *T. velifera* were highly prevalent throughout the entire study period, with no significant difference between groups. As previously discussed in Chapter 3, these findings reflect the presence of a carrier status in the juvenile and adult cattle population of the study area, characterised by a persistent detectable parasitaemia of both of these benign *Theileria* species (Bishop *et al*., 2009). The absence of significant difference in the kinetic trend of infections of the two groups proves the lack of interference of the 100% RAP protocol with the epidemiology of these two piroplasm species, both transmitted by *A. variegatum* ticks.

4.5.9 *Theileria taurotragi*

In line with results from Chapter 3, this study confirmed the presence of *T. taurotragi* in the village of Hurti. Originally associated with eland (*T. oryx*) in Southern and Eastern Africa, the occurrence of this piroplasm, in the case of Nigeria, has been put in relation with the presence of local antelopes (e.g. *Ourebia ourebi*, *Tragelaphus scriptus*, *Cephalophus rufilatus*, *Sylvicapra grimmia*, etc.) on the Plateau (Anadu, 1987). Nevertheless the tick species acting as vector of *T. tautragi* in this area still needs to be documented, considering that, at present, its two known competent vectors (i.e. *Rh. appendiculatus* and *Rh. zambeziensis*) cannot be found in Nigeria (Walker *et al*., 2000).

In the present study, *T. taurotragi* was found highly prevalent in both treated (mean: 97.7%) and control (mean: 81.8%) groups. Significant difference in the trend of infection of the two groups was found during the months of April and November 2012 (see Figure 4.15). Considering that the tick species involved in the transmission of this haemoparasite, has not yet been clarified in the study area, it is inferable that the 100% RAP might have enhanced the turnover of ticks (especially adult stages,

supposedly belonging to *Rhipicephalus* spp.) re-parasitizing the cattle, transmitting higher rates of this microorganism during the aforementioned sampling times. Alternatively, this could only be the consequence of a marked increased of the already existent (although not significantly, p=0.4) difference in herd prevalence found between the two groups at the baseline. Nevertheless, in spite of the statistically significant differences recorded in April and November, on the whole, the prevalence of *T. taurotragi* was always >90% in Group A and >70% in Group B, highlighting the well-established endemicity of this microorganism in the study area, as suggested by previous findings (see Section 3.5.5).

4.5.10 *Theileria equi*-like microorganisms

This study detected the presence of *T. equi*-like DNA in animals from both Group A and Group B (see Figures 4.3 a–e and 4.21). To the best of my knowledge, this represents the first report of the presence of this microorganism, together with *B. caballi* agent of equine piroplasmosis (de Waal, 1992; Kumar *et al*., 2009), in cattle in SSA.

Similarly to *B. caballi*, in SSA *T. equi* can be transmitted by *H. truncatum* and *R. evertsi evertsi*, the former of which was found in the Plateau in a rather conspicuous presence (see Chapter 2 and Lorusso *et al*., 2013a).

As anticipated in Chapter 3, RLB-based research on equine piroplasmosis (Nagore *et al*., 2004a) already raised the issue of possibly reviewing the methodology used to name *Theileria* and *Babesia* species on the basis of the host from which they are first isolated. Interestingly, indeed, molecular work carried out in Spain detected the presence of *T. equi* in dogs (Criado-Fornelio *et al*., 2004). The same host-pathogen association has just been confirmed by recent work carried out in the Plateau State where 3/100 (4%) dogs were found infected with *T. equi* (Adamu *et al*., 2014).

Moreover, very recently *T. equi* was found to be the predominant (7.8%) protozoan DNA detected in *Haemaphysalis longicornis* ticks collected from cattle in Korea (Kang *et al*., 2013) via amplification of the 18S rRNA gene (Battsetseg *et al*., 2001). Therefore, as for the finding of *B. caballi*, detecting the presence of this

piroplasm, of yet unknown pathogenic importance in cattle, is of epidemiological interest. Noteworthy was the very high prevalence (95%) recorded in Group A in November, while in Group B a steadier pattern was seen (see Figure 4.3 a–e). Therefore, further studies should be warranted to confirm, in a first instance a) the species identity of this microorganism by means of sequencing and then b) the relationship to its putative vector, and c) the possible effect of 100% RAP on its transmission to cattle.

4.5.11 *Ehrlichia*/*Anaplasma* spp.

In keeping with the study described in Chapter 3**,** in both Group A and Group B, a number of samples turned positive only for the *Ehrlichia*/*Anaplasma* catch-all probe, not followed by any further *Ehrlichia* or *Anaplasma* species-specific signals.

The seasonal pattern of these infections was rather different between Group A and B (see Figure 4.18). Originally very similar at the baseline (7% in Group A, 6.3% in Group B), the prevalence in the two groups became significantly different (5% in Group A and 19% in Group B, p=0.04) in April, being followed by a decline in both groups (0% in Group A and 3% in Group B) in the month of August. At this sampling time (follow up II) the prevalence was the lowest within the 11-month span of investigation, suggesting a reduction of the transmission rate (and possibly also the burden by the putative tick vector) at this time of the year.

Moreover, the prevalence recorded in both Group A (9%) and Group B (5%) at the sampling time of November was very similar to that recorded in October 2008 (6.2%). A significant difference (p=0.009) between the two groups was then seen again in February 2013 (follow up IV) when a greater number of new infections caused the prevalence of Group A (25.6%) to become higher than in Group B (6.3%) (see Figure 4.19).

In the survey from 2008, all PCR amplicons from merely *Ehrlichia*/*Anaplasma* catch-all positive samples from the whole study area yielded 100% similarity with *A. platys* (AN: KC989957.1, KF360842.1, KF576217.1; see Section 3.4.3). This was the case also for the village of Hurti, where all positive samples, where all positive samples (prevalence: 6.2%) were identified as *A. platys*.

Based on this finding, an *A. platys*-specific probe was included in the RLB assay employed in the study described in Chapter 4 (see also Table 4.3). Although this probe's oligonucleotide sequence presents a similarity of 100% with *A. platys* 16S rDNA sequences available in GenBank (e.g. Accession No. KC109446.1, KF360842.1 and KF360841.1), all positive samples detected in the study here presented did not yield any specific signal for this probe. This could be either due to the presence of another *Ehrlichia*/*Anaplasma* species being detected in this study or to the presence of nucleotide sequence variation in the strain of *A. platys* circulating in the study area. The latter hypothesis is further supported by the fact that all the *A. platys* 16S rDNA fragments sequenced in the study described in Chapter 3 did not include the nucleotide sequence of the probe employed in this study, the only so far developed for the detection of *A. platys* by RLB (unpublished, see also Table 4.3). Therefore, further sequencing efforts would be required to help fill this knowledge gap, identifying the species identity of these microorganisms.

In Section 3.5.12 the possibility for *A. platys* to be transmitted by *Rhipicephalus sanguineus* Group ticks, feeding on cattle as well as small ruminants and dogs, was discussed. Further studies aiming to verify this hypothesis, as well as understanding the implications of this TBI in cattle health, are needed. Clarifying this tick-host pathogen association may also help in comprehending the effect of the RAP on the kinetics of this TBI.

4.5.12 SFG rickettsiae – opening the 'black box'

This study ascertained the presence of several SFG rickettsiae of zoonotic importance in cattle in the village of Hurti. In the survey described in Chapter 3, *Rickettsia* 16S rDNA was not detected in any of 80 animals sampled in the month of October in the village of Hurti, where the present trial took place. This result may have been affected by the fluctuating trend of rickettsiaemia occurring in the infected vertebrate hosts (Kelly *et al*., 1991), analogously with what has also been described for *Anaplasma* spp. (Eriks *et al*., 1989).

As described in Section 4.3.9, in this survey several *Rickettsia* species-specific probes were employed in order to enable a rapid detection of possible

rickettsiae of zoonotic importance, avoiding the passages of DNA purification and sequence analysis (see also Table 4.3).

Importantly, the multiple signals recorded in all positive samples, reflect the presence of infections by multiple species (i.e. *R. conorii*, *R. helvetica*, *R. massiliae*, and *R.* (DNS14)/*raoultii*; see Figure 4.2 a–b and 4.16; Table 4.6). In these cases, confirmation of species identity by purification and sequencing of amplicons could certainly be useful, but would have likely only helped identifying the species present in the highest DNA concentration, thus 'masking' the actual status of co-infection.

To the best of my knowledge, this study provides the first report of the detection of *R. conorii*, *R. helvetica* and *R. raoultii* in cattle.

R. conorii is the causative agent of the 'Mediterranean spotted fever' (MSF) (Rovery and Raoult, 2008) and 'Israeli Spotted Fever' (ISF) (Mumcuoglu *et al*., 2002), and it is usually associated with the 'brown dog tick' *Rh. sanguineus* (Santos *et al*., 2002). Until the 1990s, before the emergence of African tick-bite fever (ATBF), it was considered the only SFG rickettsia present in Africa (Cazorla *et al*., 2008). *R. conorii israelensis* was indeed recently detected in *Rh. sanguineus* and canine blood in Central-Northern Nigeria (Kamani *et al*., 2013). Interestingly, recent experimentally infected dogs were ascertained to be able to retain *Rh. conorii* and infect ticks feeding on them, thus acting as reservoirs of this SFG rickettsia (Levin *et al*., 2012).

R. helvetica has been involved with fatal cases of myocarditis in humans (Nilsson *et al*., 1999). This rickettsia was first isolated in late 1970s from *I. ricinus* ticks collected in Switzerland and thereafter was identified in the same tick species from other European countries (Parola *et al*., 2005) and in *Ixodes ovatus* in Japan (Fournier *et al*., 2002; Ishikura *et al*., 2002). Moreover, a study conducted in Japan (Jilintai *et al*., 2008), disclosed the presence of *R. helvetica* DNA in blood samples from sika deer (*Cervus nippon yesoensis*) as well as anti-*R. helvetica* specific antibodies in cattle sera. In Africa, *R. helvetica* was reported in Algeria (Kernif *et al*., 2012), Morocco (Sarih *et al*., 2008) and Tunisia (Sfar *et al*., 2008), countries where *I. ricinus* is known to be present (Sergent and Poncet, 1937; Bailly-Choumara *et al*., 1974; Bouattour, 1987).

R. raoultii is one of the two known causative agents of tick-borne lymphoadenopathy (TIBOLA) (Parola *et al*., 2009). It was first isolated in 1999 from *Ixodes* ticks collected in Russia (Rydkina *et al*., 1999), and thereafter found in *Dermacentor reticulatus* in Germany (Dautel *et al*., 2006) and Poland (Stanczak, 2008) and in *Dermacentor marginatus* in Western Europe (i.e. France and Spain) (Mediannikov *et al*., 2008). In Africa, *R. raoultii* was isolated in the Maghreb from *D. marginatus* collected in Morocco (Sarih *et al*., 2008). None of the known vectors of *R. helvetica* and *R. raoultii* is present in SSA (Walker *et al*., 2003).

As already discussed in Section 3.5.13, in Nigeria *R. massiliae* was also detected in the blood of cattle collected in October 2008 in the Plateau and also from questing *Rhipicephalus evertsi* ticks in Oyo state (Reye *et al*., 2012). To date, in fact, *A. variegatum* and *Rh. sanguineus* Group are the only vectors of SFG rickettsiae (e.g. *Rickettsia africae* and *Rickettsia conorii israelensis*, respectively) whose presence was documented in the study area (see also Chapter 2 and Lorusso *et al*., 2013a,b; Kamani *et al*., 2013). Ascertaining the tick species involved in the transmission of the rickettiae identified from the present study should be one of the aims of any future research endeavours. Moreover, it would be pivotal to ascertain the role, whether of 'accidental' or 'reservoir' host, played by cattle in the epidemiology of these zoonotic microorganisms.

In this study, a similar kinetic trend was recorded in both treated and control groups, with a minimal difference (p=0.3) in the month November 2012, when no cases were recorded in Group A while in Group B a prevalence of 4.8% (n=3/63) was found. These findings suggest that the 0.005% deltamethrin-based RAP does not interfere with the epidemiology of SFG rickettsiae in their bovine host.

4.5.13 *Ehrlichia ruminantium*

Consistently with Chapter 3, this study also detected a minimal presence of *E. ruminantium* by means of 16S PCR-based RLB. Adding a further fragment included within the 16S rRNA gene sequence (Allsopp *et al*., 1999, see Table 4.3) did not enable the detection of a higher prevalence than in the 2008 survey. Further investigations employing other gene targets (e.g. pCS20, Faburay *et al*., 2007) would

therefore be advisable to help clarify the actual occurrence of this microorganism in the study area. Moreover, as already suggested (Faburay *et al*., 2007), xenodiagnosis on specimens of the known competent vector *A. variegatum* found feeding on cattle may prove to be a useful tool to better understand the epidemiology of this important bovine pathogen in Central Nigeria.

4.6 Conclusion

In the light of the findings elucidated in this chapter, the monthly application of 0.005% deltamethrin according to a RAP did not prevent the transmission of TBIs to cattle on the Nigerian Plateau. Moreover, the deltamethrin-based RAP also proved not to interfere relevantly with the year-round kinetics of the majority of the TBIs investigated. This is probably due to several reasons including:

- The well-established presence of several tick-borne microorganisms (i.e. *A. marginale*, *T. mutans*, *T. velifera*, *E.* sp. Omatjenne; *B. divergens*, *T. taurotragi*) for which persistently infected cattle may serve as reservoir and source of infections for ticks;
- Rapid and efficient TBI-transmission, that can be carried out even by a small number of infective ticks (Kocan, 1995);
- Capacity of infected ticks to maintain their infectivity, even when feeding on non-parasitaemic hosts, for several generations (Potgieter and Els, 1977; Callow, 1979);
- Intrinsic 'sustainable' characteristics of the RAP such as:
    a) Exclusion of animals younger than 6 months in the treatment regimen;
    b) Limited extension of the application of the pyrethroid-based solution to certain body areas of cattle;
    c) Frequency of applications of RAP, apparently minimally interfering with the life cycle of most tick species;

Nevertheless, the differences recorded between the treated and control group with regards to *B. bigemina* and *B. bovis* prevalences in August 2012, together with the significantly higher CI of *B. bovis* reported from August to February in Group A compared to B, highlight a certain sensitivity of boophilid ticks to RAP, with

inevitable consequences on the kinetics of babesiosis transmission, with special reference to *B. bovis* infections. This is of relevance, considering that farmers of the study area may report cases of clinical babesiosis during the period of the rains (Tok[15] and Santirso[16], personal communication). Would the 0.005% deltamethrin-based RAP be adopted as a routine practice on the Nigerian Plateau, babesiosis should certainly be a vector-borne disease to monitor, as its epidemiological status could be more easily disrupted, after a number of seasons of treatments. Alternatively, 'diluting' the frequency of RAP to a time span longer than one month (e.g. every two months) could perhaps reduce the rapidity of turnover of (infective) boophilid larvae on treated cattle.

This survey did not include tick counts at any of the sampling times. Civil unrest in the study area during the year of 2012 did not allow the author of this thesis to reach the sites of fieldwork and perform the tick counts from the study animals at the time they were blood-sampled (i.e. baseline and follow ups I– IV). Yet, personnel from NITR carrying out the blood sampling and treatment operations was not able to perform tick counts due to lack of expertise in tick species recognition.

Providing information on the tick burden at the species/genus level would have possibly added further evidence to the speculations raised in the Discussion section of this chapter. However, the rather similar prevalence recorded in the treated and control group with regards to *T. mutans*, *T. velifera* and *Rickettsia* spp. suggest an unchanged dynamic of their vector *A. variegatum* in both herds. In a study carried in Burkina Faso from May to the end of July 2005, the use of foot baths employing the same concentration of Vectocid® (CEVA Santé Animale, Libourne, France) proved to reduce significantly the number of *A. variegatum* and *Rhipicephalus* spp. (e.g. *Rh. lunulatus*) ticks attached to treated cattle (Stachurski and Lancelot, 2006). In the same study, a reduced, though not significantly, burden of both *H. rufipes* and

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H. truncatum, both found in the Nigerian Plateau (see Chapter 2) and implicated in the transmission of some novel microorganisms reported in this study in either cattle (i.e. *T. equi*-like) or in Nigeria (i.e. *B. caballi*). Nevertheless in the study by Stachurski and Lancelot (2006), cattle were led through the footbaths every other day. In the light of their findings, Stachurski and Lancelot (2006) proposed a 'strategic' and 'threshold' control of ticks, to be carried out during the wet season, as soon as animals bear 40 ticks/head, in order to preserve endemic stability, whilst minimizing the most harmful effect derived from heavy tick burden.

Successful management of TBIs heavily depends on adequate knowledge of their actual occurrence, seroprevalence, and the risk factors associated with transmissions (Alonso *et al*., 1992; Swai *et al*., 2005).

At present, there is lack of evidence that manipulating tick population size with acaricides can be used effectively to reduce TBIs transmission to indigenous cattle (Jonsson *et al*., 2008). The only general recommendation that can be made in these contexts regarding tick control is to treat animals when it is justified to mitigate the effects of ticks on growth and milk production or when the infestation levels are considered unacceptable on a welfare point of view (Jonsson *et al*., 2008).

Preserving endemic stability implies indeed maintaining tick populations above a certain minimum threshold in the indigenous cattle populations. This is very likely achieved employing the RAP here described. Nevertheless, in the event of importation of exotic taurine (*Bos taurus*) breeds in the study area, adoption of 'intensive' control strategies would become necessary.

Possible alternative sustainable strategies will be discussed in the following chapter.

Chapter 5 – Conclusions

This thesis aimed to assess the occurrence of ticks and TBIs in cattle from an area of Central Nigeria, the Plateau State, where extensive livestock management is practiced employing indigenous cattle. In addition, the effect on bovine TBIs of an 11-month long deltamethrin-based intervention for the control of tsetse-borne trypanosomiasis was monitored longitudinally.

Altogether, data here generated would serve to advocate tick control in this region.

5.1 Results highlights

The Fulani pastoralists from the Plateau have long considered ticks as hazardous to their livestock, preferring traditional methods to acaricidal means of control, as reviewed in **Chapter 1**.

Chapter 2 provided a major update on the existing knowledge on the ixodid fauna in Nigeria. The survey described here found that cattle were infested by a relatively high number of adult ticks (i.e. 22 ± 1.4 per animal). These counts are very likely affected by several factors such as i) the traditional 'de-ticking' practice carried out by the local pastoralists (Bayer and Maina, 1984; Maina, 1986; Otufale and Adekoya, 2012); ii) the heritable resistance of the autochthonous zebu cattle to high tick burden (Seifert, 1971; Oberem, 1984), and iii) the pasture spelling carried out by the Fulani as part of their transhumance system (Maina, 1986).

Calves were found to be significantly less infested than adult cattle in accordance with previous studies conducted in SSA on indigenous bovine breeds (Jongejan *et al*., 1987; Marufu *et al*., 2011). These findings can be justified by conformational (i.e. smaller body surface of calves compared to adults) (Mooring *et al*., 2000), behavioural (i.e. grooming of the dams) (Fivaz and de Waal, 1993) and management practices (i.e. limitation of calves' grazing in the open pastures) (Maina, 1986).

Most tick species retrieved (i.e. *Rhipicephalus* (*Booophilus*) spp., *Amblyomma variegatum* and *Hyalomma* spp.) have already been described in cattle from this country and are of great veterinary importance, due to both their direct

harmfulness when feeding and their role as vectors of pathogens. In the light of the results of this study, as well as of previous work (Dipeolu, 1975), it is likely that *Rhiphicephalus* (*Boophilus*) *decoloratus* is the principal tick parasitizing cattle throughout most of Nigeria.

This study also provides new locality records for *Rhipicephalus guilhoni* in Central Nigeria, which was found in 8/9 of the surveyed villages.

Moreover, a considerable number of ticks were identified as belonging to the *Rhiphicephalus simus* group due to their morphological features ascribable to this taxon, yet not distinctive enough to enable a resolute inclusion of these specimens in any of the three species (i.e. *Rh. muhasamae*, *Rhipicephalus praetextatus*, *Rhipicephalus simus* sensu stricto) of this group.

This study ascertained the absence of *Rh.* (*Bo.*) *microplus* from the Plateau. The progression of this tick in this part of Nigeria may be hampered by the local climatic conditions (de Clerq *et al.*, 2013).

Chapter 3 illustrated the application of a molecular tool, such as the RLB assay, to detect up to five genera (i.e. *Ehrlichia*, *Anaplasma*, *Theileria*, *Babesia* and *Rickettsia* spp.) of tick-borne haemoparasites in a sample set of 704 autochthonous cattle of several age classes in Central Nigeria. This was the first molecular survey on bovine tick-borne haemoparasites in Nigeria. For the first time, three PCRs targeting *Ehrlichia*/*Anaplasma* spp. and *Rickettsia* spp. 16S and *Theileria*/*Babesia* spp. 18S respectively, were employed together in a single cattle survey.

The resourcefulness of the molecular technologies applied was proven to be considerable. The application of the RLB assay was appropriate for the diagnosis of mixed and carrier state infections within the cattle host, thus enabling the molecular characterization of new host-pathogen relationships (i.e. *Anaplasma platys* and *Rickettsia massiliae*) as well as the occurrence of microorganisms not known to be present in Central-Northern (i.e. *A. centrale*) or the whole (i.e. *Ehrlichia* sp. Omatjenne) of Nigeria or even West Africa (i.e. *Theileria taurotragi*).

Importantly, the employment of a further PCR targeting *Rickettsia* spp., in addition to the first two targeted genera of microorganisms (i.e. *Ehrlichia*/*Anaplasma* spp. and *Theileria*/*Babesia* spp.) so far employed for screening of livestock in SSA,

enabled the detection of a zoonotic SFG rickettsia (i.e. *R. massiliae*), likely to be overlooked otherwise, in 7/9 villages.

Consistent with previous work and also the findings from Chapter 2, calves were found significantly less infected than juvenile and adult cattle.

The high prevalence (>30%) recorded for certain microorganisms (i.e. *Theileria mutans*, *Theileria velifera*, *Theileria taurotragi*, *Anaplasma marginale* and *Ehrlichia* sp. Omatjenne) suggests the presence of a scenario of well-established endemicity for these microorganisms.

The lower prevalence (i.e. 6.3%) recorded for *A. centrale* suggests a less stable epidemiological scenario for this microorganism compared to *A. marginale*.

The low prevalence (i.e. 1.1%) of *E. ruminantium*, recorded only in juvenile and adult cattle suggested the lack of acute infections.

With regards to *Babesia* spp., also known for being characterised by a carrier state in post-acute infection (Zintl *et al*., 2005), the lower prevalence recorded could be attributable to:

i) shorter duration of carrier state when compared to *Anaplasma marginale* and *Theileria mutans* (Zintl *et al*., 2005);

ii) possible chronic parasitaemia below the threshold of RLB detectability in carrier animals (Calder *et al*., 1996).

A further confirmation of the two aforementioned hypotheses could be given by the application of serological tests detecting *Babesia* spp. antibodies.

These elements should be taken into account before hypothesizing, as an alternative: iii) an epidemiologically unstable condition.

Furthermore, the RLB test employed proved also very sensitive, beyond PCR limits, for the detection of *Anaplasma marginale*.

In **Chapter 4**, the RLB assay was employed for a longitudinal survey aiming to evaluate the impact of monthly applications of 0.005% deltamethrin spray according to a RAP on the kinetics of bovine TBIs.

In this case, the array of microorganisms against which each sample was tested was expanded from 12 species of Chapter 3 to 25 species of Chapter 4.

Inevitably results contributed to a better understanding of the epidemiology of TBIs in Central Nigeria, disclosing further novel tick-host-pathogen interactions (*Babesia caballi*, *Theileria equi*-like etc.).

Moreover, being the first of this kind, this study provides useful indications that could be transferred into other geographical areas of rural SSA where pyrethroid-based tsetse control is applied to indigenous cattle.

5.2 Recommendations for a future research agenda

Results generated in this PhD thesis provide the foundation for future research aiming to better understand the epidemiology of ticks and TBIs in Central Nigeria and, more in general, in SSA.

5.2.1 Ticks

With regards to the ticks collected from cattle, several further questions arise from the work described in Chapter 2. In particular, this chapter provides the foundation for further investigations, focusing on:

I. Further examining the *Rh. simus* group, to better ascertain morphological and molecular differences occurring between the species currently ascribed within this taxon. A large-scale survey could be therefore carried out, aiming to compare 'type' specimens identified so far as *Rh. muhsamae, Rh. praetextatus* and *Rh. simus* collected in West, East, and Southern Africa respectively, recognized as zone of typical distribution of these tick species (Walker *et al*., 2000). In these regards, the use of molecular biology to target mitochondrial (e.g. 16S and 12S of the ribosomal DNA, cythocrome c oxydase sub-unit 1 gene) and nuclear (e.g. internal transcribed spacer-2 region) markers may provide useful information on the actual speciation of these ticks (Latrofa *et al.*, 2013).

II. Assessing pathogens detection in the tick specimens collected within the work described in Chapter 2. This would be of great epidemiological interest considering that the tick survey illustrated in Chapter 2, identified tick species so far

scantly investigated with regards to vector competence (e.g. *Rhipicephalus guilhoni*, *Rhipicephalus simus*, *Rhipicephalus* (*Booophilus*) *geigyi*). Moreover, it may also help better understand the epidemiology of *B. bovis* in the study area. To do so, focusing on both immature and adult ticks would be desirable with the main limitation though that identifying immature boophilid ticks to the species level may prove cumbersome (Walker *et al.*, 2003). Nonetheless, these days molecular biology-based tools can be employed to further confirm the species identity of immature boophilids (Lempereur *et al.*, 2010), overcoming the limitations of traditional morphology-based identification methods.

III. Documenting pathogens' prevalence in ticks known as competent vectors of the microorganisms ascertained to be 'endemically stable' in the Plateau State with this thesis work, may also help understand the contribution of ticks' infection rates to the establishment and maintenance of endemic stability in this area.

5.2.2 Reverse Line blotting – an instrument of capacity building?

This thesis work demonstrated both the usefulness and versatility of the reverse line blot (RLB) hybridization method towards the sensitive (up to 43 copies/µl for *A. marginale*) detection of multiple tick-borne haemoparasitic infections in cattle in a West African (Chapter 3) and a more broadly sub-Saharan African context (Chapter 4). In these settings the tools developed could be employed in livestock for:

- Epidemiological surveys in areas scantly investigated before, providing precious information for

i) The devising of possible control plans;

ii) To advice possible import of exotic taurine cattle;

iii) Assessing therapeutic approaches.

Coupling this method with serological tools could provide useful information on the epidemiological status of certain infections, bearing in mind that serology

cannot allow multiple screenings and may also be affected by cross-reactions (Pessos *et al.*, 1998).

Moreover, the RLB method could also be employed for diagnosis of other zoonotic TBIs of medical importance caused by the SFG rickettsiae, *Bartonella* spp., *Borrelia* spp. and *Coxiella burnetii*, responsible for febrile syndromes often misdiagnosed as malaria in SSA (Cazorla *et al.*, 2008; Vial *et al*., 2006).

For example, recently, a point-of-care laboratory was implemented for the screening of human samples for all possible causative agents of flu-like syndromes in rural Senegal, with the aim to overcome the lag of time existing between the testing and treating of patients (Sokhna *et al.*, 2013). In this case, samples were screened via rapid diagnostic test (RDT) for malaria and dengue fever, and, via individual qPCRs for *Tropheryma whipplei*, *Borrelia* spp., *Coxiella burnetii*, *Rickettsia conorii*, *Rickettsia africae*, *Rickettsia felis*, and *Bartonella* spp. (Sokhna *et al.*, 2013). The latter six genus or species of microorganisms mentioned can be transmitted via tick bites (Cazorla *et al*., 2008).

Out of 440 patients referred to the centre between 2011 and 2012, 54 (12%) were positive for malaria, 35 (8%) for *Borrelia crocidurae*, 30 (7%) for *R. felis*, 19 (4.3%) for *Bartonella* spp., 2 (0.4%) for Q fever (i.e. *C. burnetii*) and 1 (0.2%) for *T. whipplei* infection, showing therefore that the majority of cases (86/141, 61%) could be treated via administration of doxycycline (Sokhna *et al.*, 2013).

Using RLB in this case would have allowed the prompt simultaneous diagnosis of all the aforementioned tick-borne microorganisms within one single test.

Developing an RLB method from scratch can certainly be an expensive task. Nevertheless, having the possibility to have an ‘all-in-one’ screening approach, that can be routinely used, would enable to compensate soon the expenses.

5.2.3 Tick-borne infections

With regards to TBIs, considering the findings generated from the investigations carried out on bovine blood, future studies would need to address the following points with regards to veterinary and medical pathogens detected:

I. Epidemiological status of *T. taurotragi* in West Africa – Known to be present only in Eastern and Southern Africa due to the presence of its wildlife reservoir (i.e. *Taurotragus* (*Tragelaphus*) *oryx*) and tick vectors (i.e. *Rhipicephalus appendiculatus* and *Rhipicephalus zambesiensis*) (Young *et al.*, 1980; Lawrence *et al*., 1983), the finding of this theileria in Nigerian cattle should be followed up by the ascertainment of its vector in the area, before the identification of its vertebrate reservoir.

II. Ascertaining the vector of *A. platys* to cattle and the occurrence of this canine pathogen within the dog population of this area.

III. To further understand the actual presence of *E. ruminantium*, whose life cycle in the vertebrate host may not favour the dectability via whole blood screening, and better understand the pathogencity of *E.* sp. Omatjenne, previously appointed as responsible for 'heartwater-like' syndromes in ruminants (Du Plessis, 1990**,** Allsopp *et al*., 1997), found to be endemic in Central Nigeria.

IV. Identifying the tick species acting as vector of *Babesia divergens* in Central Nigeria and possibly assessing the occurrence of this piroplasm in the human hosts.

V. To better understand the vectors of *B. caballi* and *T. equi*-like microorganisms in the area and their occurrence in other non-bovine vertebrate hosts.

VI. To deepen the understanding of *Rickettsia* spp. circulating in the area by targeting other genes besides the ribosomal 16S (e.g. citrate synthase A; *glt*A, outer membrane protein A and B, *omp*A and *omp*B).

5.3 Perspectives for tick control

Ticks and TBIs are well established in rural SSA including the Nigerian Plateau State. Here, the local pastoralists have long been aware of the problem that

ticks represent to their cattle, undertaking traditional methods of control, such as the manual 'de-ticking', pasture spelling and burning of the vegetation around the pastures (Maina, 1986).

Their manual removal mainly targets the most conspicuous *A. variegatum* and *Hyalomma* spp. ticks, as opposed to the considerably smaller *Rhipicephalus* (*Boophilus*) spp., which are however involved in the transmission of *Anaplasma* and *Babesia* spp.

Nevertheless, it should be noticed that, besides their remarkable size and distinctive colour, *Amblyomma* and *Hyalomma* ticks also share certain localization sites such as the cattle udder (see also Sections 1.2.1.5.1 and 1.2.1.5.2, Table 1.1), area that the Fulani herders handle on a daily basis while milking their cows. This is further proved by the observation of specimens of the same species left attached to other less accessible body areas (e.g. tail switch, interdigital cleft, etc.), compared to their removal from the highly frequented regions of the animal (see Chapter 2).

In spite of their awareness of ticks, and although they do regularly administer chemical medicaments (e.g. oxytetracyclines) to their cattle (Majekodunmi, 2011), thus far Fulani have been reluctant to the use of acaricides to control ticks. This could be due to their association of acaricides with dips, which historically they did not regard favourably for keeping their livestock from grazing pastures (Leeflang and Ilemobade, 1977a).

5.3.1 Stamp Out Sammore

The 'Stamp Out *Sammore* (SOS)' programme introduced ready-to-use acaricides, based on deltamethrin, to be applied on a monthly basis in spray (0.005%) or pour on (1%) formulation to a number of villages within the Plateau State of Nigeria (see also Section 4.1). This initiative was justified by the high prevalence of bovine trypanosomiasis previously found in this area (Majekodunmi, 2011; Majekodunmi *et al*., 2013).

On the whole, the local Fulani pastoralists embraced this control programme enthusiastically. Initially motivated by the will to halt '*Sammore*', they eventually also appreciated the advantage of using spray and pour on formulations to halt tick

infestation in their cattle, especially during the wet season, when the presence of these ectoparasites becomes significant in the environment and in livestock (Olaniyan[17], personal communication). Some herders for instance looked in favour upon this operation and were willing to undertake more frequent applications, up to once a week, during the times of year with the highest challenge (Olaniyan[11], personal communication).

Now that this programme has come to an end, the Fulani have the possibility to choose whether to autonomously continue to make use of acaricides or not, whether with the aim to control trypanosomiasis, or ticks, or for both causes.

In this scenario, however, sharing our findings with the community can help their decision making, for the sake of the health of their livestock and the wealth of the pastoralists too.

5.3.2 The epidemiology of babesias – some indications to consider

The doubts risen by Chapter 3 with regards to the epidemiological status of *B. bigemina* and *B. bovis* infections were seemingly clarified by the findings of Chapter 4, suggesting the endemic presence of these two microorganisms in this area as well as *Anaplasma marginale*, *E.* sp. Omatjenne, *Theileria mutans*, *Theileria velifera*, *Theileria taurotragi* and *B. divergens*.

Looking at the results from Chapter 4, the first assumption that could be inferred from this study is that treating cattle with 0.005% deltamethrin according to a restricted application protocol (RAP) does not prevent animals cured with diminazene from being re-infected by *Babesia* spp., as seen in cattle from both Group A and B in the study village of Hurti, found re-infected with babesias, already at the follow up of August 2012.

[17] Olaniyan, Shola; PhD student conducting research under the 'Stamp out *Sammore* (SOS)' programme, Welburn Research Group, School of Biomedical Sciences, the University of Edinburgh, Edinburgh, UK.

The significantly higher prevalence of *B. bovis* and the considerably (yet not statistically significant) prevalence of *B. bigemina* registered in the treated compared to the control group in the village of Hurti, suggest a possible interference of the RAP with the kinetics of *Babesia* infections and, supposedly, with the dynamics of infestation by boophilid ticks (see also Section 4.5.5). These results should be cautiously considered, as the extended repeated use of acaricides may reproduce this effect on a larger scale, at least in the short term.

5.3.3 The influence of tick infestation upon animal health

On the whole, it is inferred that, across the study area, ticks may represent more of a hazard *per se*, rather than for the microorganisms or pathogens they transmit to the indigenous cattle, due to well-established tick-host-haemoparasite relationships, leading to a balanced state characterised by paucity of clinical signs referable to TBDs. In the light of these findings, it would be advisable to keep chemical control under at a minimum level in the study area, also to prevent the insurgence of acaricide resistance.

Nevertheless, some tick species, especially those with elongated mouthparts (i.e. *A. variegatum* and *Hyalomma* spp.), do still impair cattle fitness and productivity, due to their preferential localization to body areas like the udder. Their infestation here, may cause severe teat damage (see Figure 5.1) interfering with the dams' milk production thus with the suckling and growth of their calves.

Figure 5.1 – Significant teat damage due to massive tick infestation in the udder of cattle.

Therefore, in the light of the inefficiency of the hand-picking method and that most of the calving occurs towards the beginning of the wet season (Maina, 1986), when the abundance of adult ticks of the aforementioned species reaches its peak (Bayer and Maina, 1984), the implementation of a minimal or 'threshold' chemical control of ticks is here suggested. This would be based on the application of spray or pour on acaricides to the udder region and the groin, might help prevent the topical attachment of *A. variegatum* and *Hyalomma* ticks, thereby expectantly improving milk yields (Kaiser *et al*., 1982; Figure 5.2). In this case, it would be necessary that the concentrations of the acaricide employed (depending on its potency) is kept within a certain threshold, in order to achieve nil withholding periods for both milk and meat consumption.

This control strategy would likely be needed only during the wet season (i.e. June to October), considering the lower burdens of adults of these ticks during the rest of the year (Bayer and Maina, 1984).

Importantly, this approach would also preserve the endemic stability of the indigenous cattle herds in the area with regards to bovine TBIs (Coleman *et al*., 2001; Eisler *et al*., 2003).

However, while the indigenous White Fulani cattle are better able to bear these tick burdens, it is likely that exotic (*B. taurus*) or cross (*B. indicus* x *B. taurus*) breeds, if introduced in this area, will become heavily infested with ticks thus not being able to heritably withstand tick burdens (de Castro and Newson, 1993), at least in the short term. Moreover, being parasitized by ticks, they could become infected with a variety of highly prevalent microorganisms diagnosed in the area, and this may lead to the outbreaks of several TBDs such as anaplasmosis and babesiosis, within these imported (naive) cattle.

In this case, intensive acaricide treatment (e.g. once a week applications) would become imperative.

So far most of the Fulani pastoralists in Central-Northern Nigeria have regarded the importation of taurine breeds in the area as not cost-effective (Pullan, 1980). Considering the high prevalence of numerous TBIs found as well as their tick vectors, it is likely it would require a major economic effort only for tick control. Inevitably, such an expense investment would also have to be coupled with costs required by supplementary feeding, water provision etc.

5.4 Closing statement

In conclusion, it is advisable that funding made available for the Fulani pastoralists from the Plateau would be invested to overcome shortages of fodder and water. The traditional tick control practices in place in the area have been utilised successfully for many centuries but could be further supported by a minimal chemical input. This should be limited to milking cows during the months of wet season, to positively impact their dairy productivity, for the benefit of local communities.

Figure 5.2 – A Fulani pastoralist and his cows: a tight centenarian bond.

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6 - Appendix

Table 6.1 – Extensive format of Table 3.8. Patterns of multiple infections by tick-borne haemoparasites according to age classes and overall number of animals.
(*Ac = Anaplasma centrale*; *Am = Anaplasma marginale*; *Ap = Anaplasma platys*; *E*spO = *Ehrlichia* sp. Omatjenne; *Er = Ehrlichia ruminantium*; *R = Rickettsia* spp.; *Bb = Babesia bigemina*; *Bbov = Babesia bovis*; *Tm = Theileria mutans*; *Tt = Theileria taurotragi*; *Tv = Theileria velifera*).

	Tick-borne haemoparasite species combinations	**Frequency**			**Totals**
		Calves	**Juveniles**	**Adults**	
1	*Tm_Tt_Tv*	1	13	65	79
2	*Am_E*spO_*Tm_Tt_Tv*	1	14	27	42
3	*Tm_Tv*	-	8	26	34
4	*Am_E*spO	-	4	29	33
5	*Am_Tm_Tt_Tv*	-	9	18	27
6	*Am_E*spO_*Tm_Tv*	-	6	15	21
7	*Ap_Tm_Tt_Tv*	-	2	10	12
8	*Am_E*spO_*Tm_Tt_Tv_Bb*	-	5	7	12
9	*Am_Tm_Tv*	-	2	9	11
10	*Am_E*spO_*Tm*	-	7	3	10
11	*Am_Ac_E*spO_*Tm_Tt_Tv*	-	1	9	10
12	*E*spO_*Tm_Tt_Tv*	-	-	10	10
13	*Am_Ac*	1	1	6	8
14	*E*spO_*Tm_Tv*	-	1	7	8
15	*Tm_Tt_Tv_Bb*	-	4	4	8
16	*Ap_Tm_Tv*	-	-	7	7
17	*Am_Tm*	1	1	5	7

	Tick-borne haemoparasite species combinations	Frequency			Totals
		Calves	Juveniles	Adults	
18	*Am_Ac_E*spO	-	2	5	7
19	*Am_Ac_Tm_Tt_Tv*	-	3	3	6
20	*Tm_Tt*	-	5	1	6
21	*E*spO_*Tm*	-	4	1	5
22	*E*spO_*Tm_Tv_Bb*	-	5	-	5
23	*Am_Ac_Tm*	1	2	1	4
24	*Am_Tm_Tt_Tv_Bb*	1	3	-	4
25	*Am_Ac_Tm_Tv*	-	-	3	3
26	*Am_E*spO_*Tm_Tt_Tv_Bbov*	-	1	2	3
27	*E*spO_*Bb*	-	2	1	3
28	*E*spO_*Tm_Tt*	-	2	1	3
29	*E*spO_*Tm_Tt_Tv_Bb*	-	-	3	3
30	*E*spO_*Tm_Tt_Tv_Bbov*	-	1	2	3
31	*R_Tm_Tv*	-	2	1	3
32	*Tm_Tt_Bb*	-	2	1	3
33	*Tm_Tv_Bb*	1	1	1	3
34	*Ap_Tm*	-	1	1	2
35	*Ap_Tm_Tt*	-	-	2	2
36	*Ap_Tm_Tt_Tv_Bb*	-	-	2	2
37	*Ac_E*spO	-	-	2	2

	Tick-borne haemoparasite species combinations	Frequency			Totals
		Calves	Juveniles	Adults	
38	*Am_Bb*	2	-	-	2
39	*Am_Ac_E*spO_*Tm*	-	-	2	2
40	*Am_Ac_Er_Tm_Tt_Tv*	-	-	2	2
41	*Am_Tm_Tt_Tv_Bbov*	-	1	1	2
42	*Am_E*spO_*Tm_Bbov*	1	-	1	2
43	*Am_Ac_E*spO_*Tm_Tv*	-	-	2	2
44	*Am_E*spO_*Tm_Tt*	-	2	-	2
45	*Am_E*spO_*Tm_Tt_Bb*	-	1	1	2
46	*Am_Ac_Er_E*spO_*Tm_Tv*	-	1	1	2
47	*Am_R_Tm*	-	2	-	2
48	*Ac_Tm_Tt_Tv*	-	2	-	2
49	*E*spO_*R_Tm_Tv*	-	-	2	2
50	*Tm_Bb*	1	-	1	2
51	*Tm_Tt_Tv_Bbov*	-	-	2	2
52	*Ap_Tm_Tt_Bb*	-	1	-	1
53	*Ap_Tm_Tv_Bb*	-	-	1	1
54	*Am_Ac_E*spO_*Tv*	1	-	-	1
55	*Am_Ac_E*spO_*Bb*	1	-	-	1
56	*Am_Ac_Er_E*spO	-	1	-	1
57	*Am_Ac_Tm_Bb*	1	-	-	1

6.1 History of reverse line blotting (RLB)

This Section provides an overview of the history of the development of the reverse line blotting (RLB) method as well as its application to date towards the detection of TBIs, with special reference to SSA.

6.1.1 1995 to date, from The Netherlands to the world

Initially developed in the mid 1990s, for the identification of *Streptococcus* serotypes (Kaufhold *et al*., 1994), and, further on, for the differentiation of *Mycobacterium tubercolosis* strains (Kamerbeek *et al*., 1997), the RLB permits the screening of multiple samples for multiple microorganisms. The novelty of this approach resided in the hybridization of PCR products with species-specific probes immobilized on a blotting membrane.

The first application of the RLB technique for the diagnosis of TBIs was developed in 1995 in a laboratory at the National Institute of Public Health and the Environment, in Bilthoven, The Netherlands (Rijpkema *et al*., 1995). The initial assay could detect up to four *Borrelia* species at the same time using the 5S–23S rDNA intergenic spacer as a target region, with a nested PCR protocol being performed prior to the RLB (Rijpkema *et al*., 1995). Initially used for the screening of *Ixodes ricinus* ticks (Rijpkema *et al*., 1995), this approach was soon applied to field epidemiological studies carried out on ticks in Ireland (Kirstein *et al*., 1997) and bird and rodent reservoirs in England (Kurtenbanch *et al*., 1998).

Afterwards, a major push forward in the development of this technique for the diagnosis of TBIs in livestock was represented by the implementation of an assay allowing detection and differentiation of all known *Theileria* (*T. annulata*, *Theileria parva*, *T. mutans*, *T. taurotragi*, *T. velifera* and *Theileria orientalis* group) and *Babesia* (i.e. *B. bigemina*, *B. bovis*, *B. divergens*) species of importance to cattle health in tropical and sub-tropical settings (Gubbels *et al*., 1999). This work derived from a collaboration between the Dutch National Institute of Public Health and Environment and the Utrecht Centre for Tick-borne Diseases (UCTD), at the Faculty of Veterinary Medicine of Utrecht, The Netherlands, and the Department of Pharmacology at the University of Granada, Spain. In this case, the conserved

domains of the 18S rRNA gene shared by both *Theileria* and *Babesia* species were targeted in order to amplify the hypervariable V4 region enclosed within the common edges (Gubbels *et al*., 1999). The discrimination between species was therefore based on differences present within the 18S rRNA V4 hypervariable region of *Theileria* and *Babesia* spp. Within these regions, short species-specific fragments of ~15–20 nucleotides were deduced, in order to design species-specific oligonucleotide probe (Figure 6.1). Moreover, genus-specific (i.e. 'catch all') probes were designed using a 19 nucleotide-long fragment present within all nine species' 18S rRNA V4 hypervariable region. At that time, for most species, one or more 18S rRNA sequences were available in GenBank, while for designing of *T. velifera* 18S rRNA specific fragment, the authors referred to original samples (Gubbels *et al*., 1999).

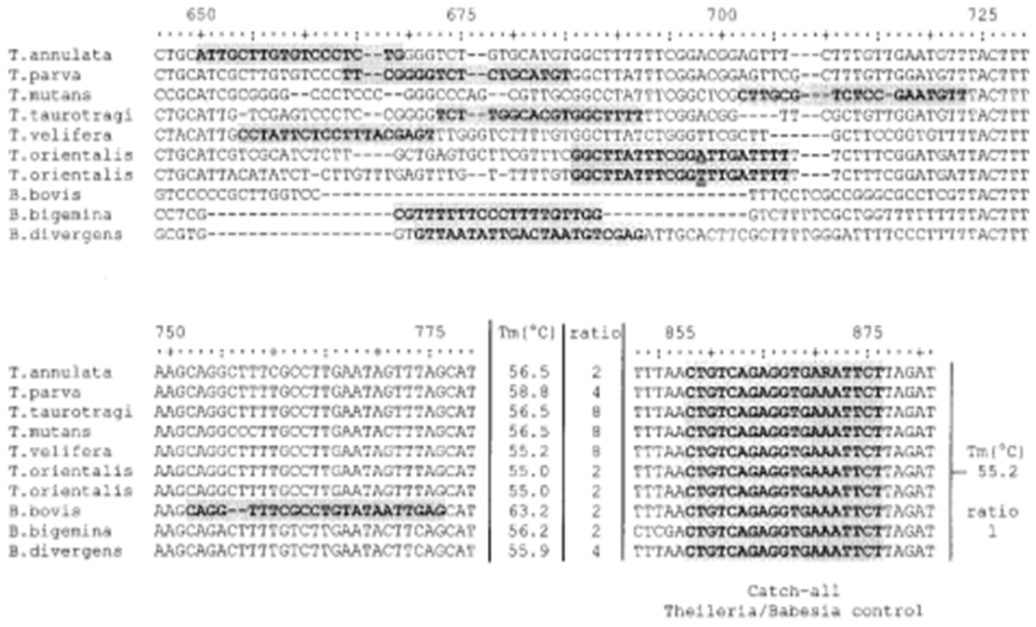

Figure 6.1 – Locations of species-specific and 'catch-all' oligonucleotides (shaded) in the 18 rRNA V4 hypervariable region of *Theileria* and *Babesia* spp.

The first *T. orientalis* isolate listed is type D (with adenine, 'A' at position 668), and the second is the *T. orientalis* isolate described as *T. buffeli* Warwick (double-underlined thymine, 'T' at position 668). The melting temperature (Tm) of each oligonucleotide is indicated. The ratio of each oligonucleotide to the 'catch-all' *Theileria* and *Babesia* oligonucleotide (a ratio of 1 corresponds to 200 pmol) is also indicated. The 'catch-all' *Theileria* and *Babesia* oligonucleotide is identical for all 10 sequences. The

'R' at position 871 in the *T. annulata* sequence denotes either adenine, 'A', or guanine, 'G' [From Gubbels *et al.* (1999), page 1783].

This method proved very useful and was soon applied to the integrated diagnosis of bovine theileriosis and babesiosis in Sicily, Italy (Sparagano *et al*., 2000). In the same year, another RLB method was developed in The Netherlands, still at the National Institute of Public Health and the Environment, enabling the simultaneous detection of a variety of *Ehrlichia* and *Bartonella* species as well as *Borrelia burgdorferi* (Schouls *et al*., 1999).

In 2002, another RLB method was implemented to detect and differentiate *Anaplasma* and *Ehrlichia* species known to occur in ruminants and *A. variegatum* ticks in the "(sub)tropics[18]" (Bekker *et al*., 2002). In this case, conserved domains within the 16S rRNA gene were used to amplify the V1 hypervariable region (Bekker *et al*., 2002). For their PCR amplification, Bekker *et al*. (2002) employed the forward primer (i.e. '16S8FE') previously designed (Schouls *et al*., 1999) and an edited version of the 'B-GA1B' as reverse (Schouls *et al*., 1999). At the same time, Georges *et al.* (2001) provided an update of the RLB technology implemented by Gubbels *et al.* (1999), designing new 18S PCR primers (i.e. 'RLB-F2' and 'RLB-R2') and a number of additional species-specific probes for detecting *Theileria* and *Babesia* species, and combined them with the *Ehrlichia*/*Anaplasma* RLB previously implemented (Bekker *et al*., 2002). Only one year after, another study (Christova *et al*., 2003) added an RLB for the detection of *Rickettsia* spp., to the protocol set up by Schouls *et al*. (1999). In the same year, in Switzerland another assay was set up to distinguish each known European *B. burgdorferi* sensu lato species (Godfroid *et al*., 2003).

In 2004, the first RLBs targeting *Theileria* and *Babesia* parasites of small ruminants were implemented. One represented the outcome of an international collaboration between research groups at UCTD, Borstel (Germany) and the Chinese Academy of Sciences (Schnittger *et al*., 2004), and included species also involved in

[18] Original word from the article by Bekker *et al*. (2002).

ovine piroplasmosis in Asia. Another protocol was designed at the Department of Animal Health at the Institute 'Vasco de Investigación y Desarrollo Agrario Berreaga' in the Basque region in Spain, to be used for the screening of sheep populations in northern Spain (Nagore *et al*., 2004b). The same group published, in the same year, a *Theileria*/*Babesia* 18S rRNA gene-based RLB protocol for the diagnosis of equine piroplasmosis (i.e. *B. caballi*, *B. caballi*-like, *T. equi*, *T. equi*-like specific probes) (Nagore *et al*., 2004a); for the first time, a single (instead of two) genus (i.e. *Theileria* spp.)-specific probe was employed. In their study, however, Nagore *et al.* (2004a) could not design a *Babesia*-specific probe "due to the great heterogeneity of the V4 hypervariable region" of this genus. Only one year after, this issue was overcome, with the design of a *Babesia* catch-all probe (Matjila *et al*., 2005), this was later followed by two other variants *Babesia* catch-all 1 and catch-all 2 (Nijhof *et al*., unpublished). In 2006, another protocol for the differentiation of *A. centrale* and *A. marginale* in Israel was set up, employing newly designed *Ehrlichia*/*Anaplasma* 16S primers (Molad *et al*., 2006).

Moreover, in 2008, the first (catch-all) probe specific for the genus *Hepatozoon* was developed as part of a survey aiming to detect canine TBIs in South Africa (Matjila *et al*., 2008a), using the upgraded version of *Theileria*/*Babesia* 18S primers provided by Georges *et al.* (2001).

In more recent times, the RLB method has been employed for the detection of borreliae in *Ixodes scapularis* ticks from North-eastern USA (Wormser *et al*., 2008; MacQueen *et al*., 2012).

Interestingly, in 2003, the RLB method was applied to the identification of host alongside pathogen DNA in *I. ricinus* ticks (Pichon *et al*., 2003). The first protocol, set up at the University College in Dublin, employed probes based on the 18S rDNA sequences of vertebrate species (Pichon *et al*., 2003).

Inevitably, this novel application of the RLB soon inspired other research groups. In 2007 researchers at the University of Neuchâtel, Switzerland, developed an RLB method to detect and differentiate vertebrate hosts on which *I. ricinus* ticks had fed, using 12S rDNA as the genetic marker (Humair *et al*., 2007; Morán Cadenas e*t al*., 2007a).

In 2010 work carried out at Washington University, USA, employed the same approach used by Pichon *et al*. (2003) to identify reservoir hosts for *Amblyomma americanum* (Allan *et al*., 2010). Similarly, another assay based on the amplification of the 12S mitochondrial rDNA of mammal, bird and reptile species was recently developed in the USA (Centre for Wildlife Health, the University of Tenessee) to identify preferential blood meal sources amongst the hosts known to be parasitized by certain ticks (Scott *et al*., 2012). Interestingly, all these assays enabled to ascertain multiple bloodmeals for feeding and questing ticks (Humair *et al*., 2007; Morán Cadenas *et al*., 2007a; Allan *et al*., 2010; Scott *et al*., 2012).

As just described, since its first implementation in 1995, RLB has proved to be a versatile technique for the assessment of tick-borne infections in both vertebrate and arthropod hosts (UCTD, 2013), rapidly becoming a standard tool for epidemiological and diagnostic studies focusing on either ticks or animals, carried out in a progressively increasing number of countries across Europe [i.e. Bulgaria (Christova *et al*., 2003), Czech Republic (Spitalská *et al*., 2011), Estonia (Katargina *et al*., 2011), France (Bonnet *et al*., 2013; Pisanu *et al*., 2014), Hungary (Hornok *et al*., 2013), Ireland (Kirstein *et al*., 1997, Pichon *et al*., 2003), Italy (Sparagano *et al*., 2003; Torina *et al*., 2010), Portugal (Baptista *et al*., 2004), Romania (Coipan and Vladimirescu, 2010, 2011; Kalmár *et al*., 2013), Russia (Alekseev *et al*., 1999, 2001), Slovakia (Smetanová *et al*., 2007), Spain (Nagore *et al*., 2004a,b; Barandika *et al*., 2007; García-Sanmartín *et al*., 2008), Switzerland (Burri *et al*., 2007, 2014; Morán Cadenas *et al*., 2007a,b; Gern *et al*., 2010; Gigandet *et al*., 2011; Huegli et al., 2011; Hermann and Gern, 2012, 2013; Hermann *et al*., 2013) and The Netherlands (Wieilinga *et al*., 2006; Nijhof *et al*., 2007; Butler *et al*., 2008, 2012; Hofuis *et al*., 2013)] and the rest of the world including Asia [i.e. Turkey (Altay *et al*., 2008, 2012; Aktas, 2014; Aktas *et al*., 2012; Aydin *et al*., 2013), Israel (Molad *et al*., 2006), China (Niu *et al*., 2012) and Pakistan (Iqbal *et al*., 2013)], North America (Wormser *et al*., 2008; MacQueen *et al*., 2012), Northern (i.e. Tunisia) (M'ghirbi *et al*., 2010; Ros-García *et al*., 2011) and SSA.

With particular regards to bovine TBIs, after the implementation (Gubbels *et al*., 1999) and update (Georges *et al*., 2001) of *Theileria*/*Babesia*- as well as the set up of *Ehrlichia*/*Anaplasma*-targeted RLBs (Schouls *et al*., 1999; Bekker *et al*.,

2002), numerous studies, based on the application of either one or the two assays in combination were carried out in European countries such as Italy (Sparagano *et al*., 2000; Georges *et al*., 2001; Torina *et al*., 2007; Ceci *et al*., 2014), Portugal (Silva *et al*., 2010), the Balearic Islands (Almería *et al*., 2002, 2009; Ros-García *et al*., 2012b) and continental Spain (Gubbels *et al*., 2000; García-Sanmartín *et al*., 2006), Turkey (Altay et al., 2007; Aktas *et al*., 2011), Asia [i.e. Israel (Molad *et al*., 2006), Pakistan (Khan *et al*., 2013) and China (Niu *et al*., 2011)] and Africa.

6.1.2 RLB in sub-Saharan Africa

Given the importance of TBIs in SSA, the development and progressive improvement of the RLB assay has played a major contribution to the understanding of the epidemiology of these infections as well as the competence of their respective vectors across several African countries.

The first RLB assay developed for TBIs (Gubbels *et al*., 1999) included, in fact, *Theileria* and *Babesia* species of great veterinary relevance in Africa, both north and south of the Sahara. Improving the diagnostics of TBIs in tropical and sub-tropical settings, was indeed one of the ideals with which this methodology was developed (Gubbels *et al*., 1999). While tests enabling the detection only of *Anaplasma* and *Babesia* species are most suited for Central and Southern America where bovids do not harbor theileridae (Criado-Fornelio *et al*., 2009), diagnostic tools dealing with cattle samples from SSA should certainly take into account *Theileria* species (for more information see Section 1.2.2.1.5).

The great suitability of this technique within this context can be essentially attributed to two reasons: a) the frequent scenario of co-infections, from which the need for an 'integrated' diagnostic approach arises, and b) the endemicity of numerous TBIs characterised by chronically infected/sub-clinical/carrier animals, for which high sensitivity tools are needed given their rather low parasitaemiae. Therefore, the assay developed a few years later by Bekker *et al.* (2002), included *Ehrlichia*/*Anaplasma* species of importance in SSA (i.e. *E. ruminantium*).

To date, these two RLB assays (Gubbels *et al*., 1999; Bekker *et al*., 2002), have been employed, either alone, i.e. *Theileria*/*Babesia* spp. (Gubbels *et al*., 2000;

Nijhof *et al*., 2003; Matjila *et al*., 2004, 2008c; Ali *et al*., 2006; Salih *et al*., 2007, 2010; Oosthuizen *et al*., 2008, 2009; Köster *et al*., 2009; Bhoora *et al*., 2009, 2010; Bosman *et al*., 2010, 2013; Schoeman and Herrtage, 2008; Penzhorn *et al*., 2008; Muhanguzi *et al*., 2010; Yusufmia *et al*., 2010; Brothers *et al*., 2011; Chaisi *et al*., 2011; Govender *et al*., 2011; Oura *et al*., 2011; Githaka *et al*., 2013; Goddard *et al*., 2013) or i.e. *Ehrlichia/Anaplasma* spp. (Faburay *et al*., 2007; Muhanguzi *et al*., 2011) or in combination (i.e. *Theileria*/*Babesia* spp. + *Ehrlichia/Anaplasma* spp.) (Oura *et al*., 2004, 2011; Matjila *et al*., 2008a,b; Martins *et al*., 2010; Tomassone *et al*., 2005, 2012; Tonetti *et al*., 2009; Pfitzer *et al*., 2011; Asiimwe *et al*., 2013; Adamu *et al*., 2014; Berggoetz *et al*., 2014a,b**)**, for epidemiological and diagnostic purposes in a number of SSA countries including Uganda (Oura *et al*., 2004, 2011; Muhanguzi *et al*., 2010; Asiimwe *et al*., 2013), the Republic of Guinea (Tomassone *et al*., 2005), The Gambia (Faburay *et al*., 2007), Sudan (Gubbels *et al*., 2000; Ali *et al*., 2006) and South Sudan (Salih *et al*., 2007, 2010), Ethiopia (Tomassone *et al*., 2012), Kenya (Githaka *et al*., 2013), Mozambique (Martins *et al*., 2010), Namibia (Penzhorn *et al*., 2008), South Africa (Nijhof et al., 2003; Matjila et al., 2004, 2008a,b,c; Schoeman and Herrtage, 2008; Oosthuizen et al., 2008, 2009; Bhoora et al., 2009, 2010; Köster *et al*., 2009; Tonetti *et al*., 2009; Bosman et al., 2010, 2013; Yusufmia et al., 2010; Brothers *et al*., 2011; Chaisi *et al*., 2011; Govender *et al*., 2011; Pfitzer *et al*., 2011; Goddard *et al*., 2013; Berggoetz *et al*., 2014a, b) and Nigeria (Ogo *et al*., 2012; Adamu *et al*., 2014).

Work carried out in the African continent, not only focused on the detection of TBIs in livestock such as cattle (Gubbels *et al*., 2000; Oura *et al*., 2004, 2011; Ali *et al*., 2006; Salih *et al*., 2007, 2010; Martins *et al*., 2010; Muhanguzi *et al*., 2010, 2011; Yusufmia *et al*., 2010; Asiimwe *et al*., 2013), small ruminants (Tomassone *et al*., 2012), horses (Bhoora *et al*., 2009, 2010), camels (Tomassone *et al*., 2012), dogs (Matjila *et al*., 2004, 2008a; Schoeman and Herrtage, 2008; Köster *et al*., 2009; Goddard *et al*., 2013; Adamu *et al*., 2014) and cats (Bosman *et al*., 2013), but also in wildlife and game animals such as the African buffalo (*Syncerus caffer*) (Chaisi *et al*., 2011), black (*Dicero bicornis*) (Nijhof *et al*., 2003; Penzhorn *et al*., 2008) and white rhinoceros (*Ceratotherium simum*) (Govender *et al*., 2011), nyala (*Tragelaphus angasii*) (Oura *et al*., 2011; Pfitzer *et al*., 2011; Berggoetz *et al*.,

2014a), roan (*Hippotragus equinus*) (Nijhof *et al*., 2005; Oosthuizen *et al*., 2009), sable (*Hippotragus niger* Harris, 1838) (Nijhof *et al*., 2005; Oosthuizen *et al*., 2008) and tsessebe (*Damaliscus lunatus*) (Brothers *et al*., 2011) antelope, greater kudu (*Tragelaphus strepsiceros*) and gray duiker (*Sylvicapra grimmia*) (Njhof *et al*., 2005), giraffes (*Giraffa camelopardalis tippelskirchi* and *Giraffa camelopardalis reticulata*) (Oosthuizen *et al*., 2008; Githaka *et al*., 2013), African wild dog (*Lycaon pictus*) (Matijlla *et al*., 2008b) and cheetah (*Acinonyx jubatus*) (Bosman *et al*., 2010). Moreover, a few other studies also focused on the detection of microorganisms in ticks, to assess pathogen detection (Tomassone *et al*., 2005; Faburay *et al*., 2007) and vectorial competence (Berggoetz *et al*., 2014b).

In South Africa, the application of the RLB has been largely employed in epidemiological surveys focusing on wildlife species, enabling the identification of novel species (i.e. *Babesia bicornis* and *Theileria bicornis*) (Nijhof *et al*., 2003) as well as contributing to the better understanding of epidemiological relationships occurring at the game/livestock interface (Yusufmia *et al*., 2010; Berggoetz *et al*., 2014a). In 2007, the RLB method was employed for the first time for the longitudinal monitoring of animals in a six month-long field trial designed to evaluate the prophylactic efficacy of 9% amitraz-impregnated collars (Preventic, Virbac, Carros, France) against canine babesiosis (*Babesia canis rossi*) in South Africa (Last *et al*., 2007).

With regards to West Africa, to date the RLB has only been employed on studies focusing on ticks collected from cattle in Guinea (Tomassone *et al*., 2005) The Gambia (Faburay *et al*., 2007) and Nigeria (Ogo *et al*., 2012; Adamu *et al*., 2014). The former two studies focused specifically on *A. variegatum* ticks, against *Ehrlichia*/*Anaplasma* and *Theileria*/*Babesia* probes (Tomassone *et al*., 2005), and to assess their vectorial capacity and suitability for xenodiagnosis of *E. ruminantium* infection (Faburay *et al*., 2007).

The two studies carried out in Nigeria focused on ticks from three species [i.e. *A. variegatum*, *Rh.* (*Bo.*) *decoloratus* and *Rh. sanguineus*] (Ogo *et al*., 2012) and dog's blood samples (Adamu *et al*., 2014), screened against *Theileria*/*Babesia* and *Ehrlichia*/*Anaplasma* probes (see Section 3.5.1). Both studies included samples collected in the Plateau State.

The present treatise highlights the versatility of the RLB method as an epidemiological and diagnostic tool, to detect both clinically apparent and sub-clinical infections (Nagore *et al*., 2004a) and for the monitoring of the post-therapeutic progression of infections (Wijnveld[19], personal communication). To the best of my knowledge, at present the RLB method is employed as a routine diagnostic method at the UCTD, Utrecht, The Netherlands, recently (i.e. November 2013) appointed as a reference center for veterinary tick-borne diseases by the Food and Agriculture Organization (FAO) of the United Nations[20], and the Onderstepoort Research Institute, Pretoria, South Africa (Matjila *et al*., 2007; Schoeman and Herrtage, 2008; Goddard *et al*., 2013). Notably, at both institutes, the RLB is also employed as a prognostic tool for the evaluation of therapeutical protocols (Matjila *et al*., 2008a; Wijnveld[19] personal communication).

6.2 Advantages of the RLB

The advantages deriving from the employment of the RLB for the detection and differentiation of TBIs can be summarized by the points here below.

6.2.1 High sensitivity

RLB has proved to be a very sensitive test for a number of microorganisms, being able to detect parasitaemiae beyond the detection limit of standard PCR (Gubbels *et al*., 1999; Nagore *et al*., 2004a; Oura *et al*., 2004; Schnittger *et al*., 2004; Molad *et al*., 2006; see also Sections 3.4.9 and 3.5.18).

[19] Wijnveld, Michiel; Laboratory manager at the Utrecht Centre for Tick-borne Diseases (UCTD), Utrecht University, Utrecht, The Netherlands.

[20] Press source: http://www.uu.nl/faculty/veterinarymedicine/EN/Current/facultynews/Pages/Utrecht-Centre_Tick_borne_Diseases_designated_FAO_Reference_Centre.aspx.

6.2.2 High specificity

By using species-specific oligonucleotide probes, RLB enables the detection and differentiation of species of tick-borne microorganisms infecting pets, livestock and humans. Absence of cross-reactions has been well demonstrated (Gubbels *et al*., 1999; Schouls *et al*., 1999; Bekker *et al*., 2002; Schnittger *et al*., 2004; Nagore *et al*., 2004a,b). Theoretically, competition for the primer volumes available in the PCR mastermix can occur among species of the same genus. However, this was shown to be prevented including a sufficiently high volume of primers in the PCR mixture (Gubbels *et al*., 1999; Schnittger *et al*., 2004)).

6.2.3 Immediacy / Cost reduction

As already said, the RLB enables the screening of multiple samples against multiple microorganisms at the same time. With special regards to TBIs, at present, the RLB allows the analysis of multiple samples (i.e. PCR products) for up to seven genera (i.e. *Bartonella*, *Borrelia*, *Ehrlichia*, *Anaplasma*, *Theileria*, *Babesia*, and *Rickettsia* spp.) of tick-borne microorganisms at the same time. Theoretically, up to 45 samples could be screened per RLB at the same time, depending on the blotter employed (for the membrane preparation and loading of the samples). It is therefore inferable that, when screening several biological samples for several tick-borne haemoparasites at the same time, the use of the RLB allows a great cost reduction compared to the application of single species-specific PCR or even a few mPCR assays. Furthermore, each membrane with the same probes attached can be re-used at least 20 times after removing the amplified products (Gubbels *et al*., 1999; Sparagano *et al*., 2000).

6.2.4 Epidemiological usefulness

The great epidemiological usefulness of the RLB when dealing with TBIs derives from two main features essentially: a) the possibility to detect co-infections and b) the opportunity to detect unexpected or novel species thanks to the presence of catch-all probes.

In the light of the feature (a), the RLB becomes the ideal tool for surveys dealing with areas of scantly or previously absent investigation or in presence of high diversity of tick species (e.g. tropical and sub-tropical settings) likely responsible for the transmission of multiple microorganisms. In such contexts, where animals may be infected by several pathogens, a test like the RLB can be also pivotal if used chronologically, to assess the progression of infections over the seasons and/or also in response to certain treatment regimens. Importantly, the availability of standardized membranes for a fixed number of tick-borne pathogens makes it possible to carry out comparative epidemiological studies between different laboratories (EMBO and ICTTD, 2003).

With regards to the feature (b), as anticipated in Section 3.3.4.3.1 the presence of catch-all probes may lead to the identification of species or genotypes of parasites not included in the initial RLB assay. Results in this case would appear as a signal on the catch-all probe, not followed by any other bands (see Figure 6.2). In this case, PCR products of these samples should be purified and sequenced to confirm species identity of these amplicons. A species-specific probe could be then designed according to the results of sequence analysis.

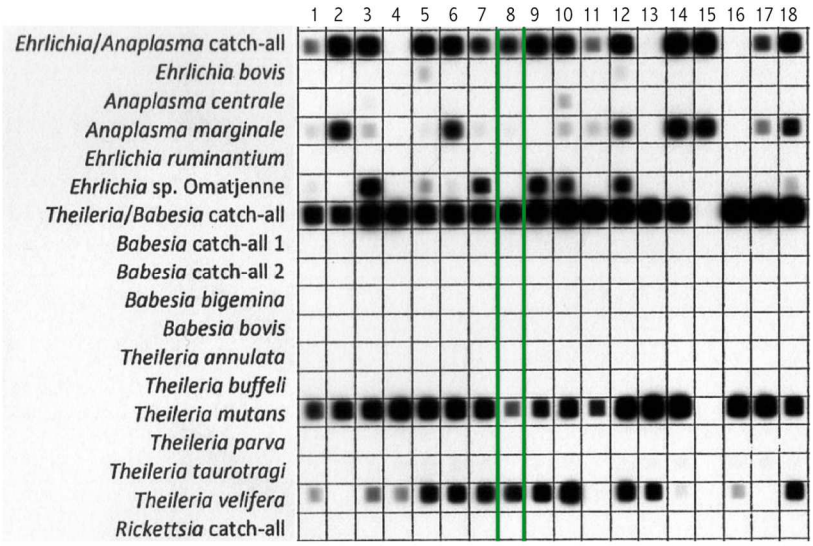

Figure 6.2 – RLB development showing one sample positive only for a 'catch-all' probe.

In this case, sample (i.e. #8) was detected positive only for the *Ehrlichia*/*Anaplasma* catch-all probe without any further species-specific signal. Further sequence analysis revealed the similarity of

this sample with *Anaplasma platys* 16S DNA fragment (see Section 3.4.5), for which a specific probe was therefore included in the membrane design described in section 4.3.9 (Table 4.3).

This could either lead to the identification of completely novel species or to a review of existing general knowledge with regards to long-established parasite–host relationships (Nagore *et al*., 2004a). At present, the former instance is more likely to occur in wildlife species, so far poorly investigated on TBIs. Accordingly, with special reference to SSA, several RLB-based studies have so far enabled the identification of novel species in South Africa, including *Babesia bicornis* and *Theileria bicornis* in black rhinos (Nijhof *et al*., 2003), *Theileria* spp. associated with mortality of sable and roan antelope, greater kudu and common gray duiker (Nijhof *et al*., 2005; Oosthuizen *et al*., 2008); *Theileria* sp. in dogs (*Canis lupus familiaris*) (Matjila *et al*., 2008c), *Babesia* sensu stricto and *Theileria* sensu stricto in giraffe and roan antelope (Oosthuizen *et al*., 2009), *Babesia lengau* sp. nov. in cheetah in South Africa (Bosman *et al*., 2010).

With regards to new pathogen-vertebrate host associations, for example, very recent work also carried out in South Africa ascertained the aetiological involvement of *B. lengau*, first characterised in cheetah also via RLB (Bosman *et al*., 2010), in two cases of cerebral and haemolytic babesiosis of domestic cats (Bosman *et al*., 2013). Results from Chapter 3 and 4 of this thesis further confirmed the suitability of the RLB in these regards.

RLB can also help document new arthropod host-pathogen associations, providing new insights on possible vector capacity of ticks (Berggoetz *et al*., 2014b). As an example, the first evidence of *Babesia occultans*, a species thought to be confined to Southern Africa (Gray and de Vos, 1981), in *Hyalomma* ticks from Northern Africa (i.e. Tunisia) was made possible via the RLB (Ros-García *et al*., 2011).

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Parasites & Vectors

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Ixodid ticks of traditionally managed cattle in central Nigeria: where *Rhipicephalus* (*Boophilus*) *microplus* does not dare (yet?)

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Abstract

Background: Ticks and tick-borne diseases (TBDs) undermine cattle fitness and productivity in the whole of sub-Saharan Africa, including Nigeria. The aim of this study was to document the composition of tick species, assessing the burden of infestation, in traditionally managed cattle in an area of central Nigeria where acaricides have not been used historically.

Methods: The study was carried out in September 2010 in 9 villages belonging to three neighbouring local government areas in Plateau State, Nigeria. In each village all visible adult ticks were collected from at least 15 cattle (mean number = 25). Collected ticks were preserved in 70% ethanol to be counted and morphologically identified to the species level.

Results: A total of 5011 ixodid ticks (1935 males and 3076 females) were collected from 228 cattle, comprising 14 calves, 33 juveniles, and 181 adults. Three tick genera (i.e., *Amblyomma*, *Hyalomma*, and *Rhipicephalus*, including the *Boophilus* sub-genus) and 11 species were identified. The most prevalent species was *Rhipicephalus* (*Boophilus*) *decoloratus* (41.4%), followed by *Rhipicephalus* (*Boophilus*) *annulatus* (15.4%), *Rhipicephalus guilhoni* (12.0%), *Rhipicephalus* (*Boophilus*) *geigyi* (7.6%), *Hyalomma truncatum* (7.4%), *Amblyomma variegatum* (6.3%), *Rhipicephalus simus* Group (4.0%), *Rhipicephalus turanicus* (1.2%), *Rhipicephalus sanguineus* (0.3%), *Hyalomma rufipes* (0.2%), and *Rhipicephalus lunulatus* (n = 1). Mean tick loads recorded were relatively high (22 ± 1.4), in spite of the practice of hand removal of ticks traditionally undertaken by the Fulani pastoralists in the area. Calves bore a significantly lower tick burden than adults ($p = 0.004$). *Rhipicephalus* (*Boophilus*) *microplus* was not found in the area, suggesting that the eastbound expansion of this tick species in West Africa, has not yet reached central Nigeria.

Conclusions: This study ascertained the presence of a broad variety of cattle tick species, most of which are of veterinary importance. The presence of each tick species is correlated with the potential occurrence of tick-borne pathogens and suggestions for tick control in the area are considered. Results should assist the diagnosis of related TBDs in cattle as well as the strategic planning of cost-effective tick control.

Keywords: Cattle, Ticks, Tick-borne diseases, sub-Saharan Africa, Nigeria

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Background

Ticks are ranked as the most economically important ectoparasites of livestock in the tropics, including sub-Saharan Africa (SSA) [1]. Their veterinary importance is related to their blood-feeding, from which both their direct and indirect pathogenicity originates [2]. In cattle, tick infestation alone can cause anaemia, stress, reduction in weight gain and milk yields, depreciation of hide value, hypersensitivity and toxicosis, leading also to secondary infections [2]. In addition, some tick species can act as vectors of pathogens causing a number of tick-borne diseases (TBDs), a serious impairment to cattle health and productivity in SSA [3].

In Nigeria, 90% of the cattle population is kept under the traditional pastoral husbandry of Fulani herders; mostly concentrated in the central-northern part of the country [4]. Under the Fulanis' management, cattle are extensively grazed in pastures and forest, and exposed to infestation by the three tick genera present in Nigeria (i.e., *Amblyomma*, *Hyalomma*, and *Rhipicephalus* spp., sub-genus *Boophilus* spp. included) [4-7]; genera are known vectors of the causative agents of the most important bovine TBDs in West Africa: anaplasmosis, babesiosis, ehrlichiosis (cowdriosis) [8]. Usually low in the dry season, tick loads on cattle tend to increase after the first scattered rains, reaching the highest abundance one month after the heavy rains (i.e., from July to September), when all tick species are expected to be present [7,9-11]. The associated tick-borne infections are endemic in the indigenous (*Bos indicus*) cattle population [8,12], and are responsible for chronic rather than acute disease symptoms. Nevertheless, TBDs may become clinically apparent in particular circumstances of malnutrition or debilitation by a concurrent disease (e.g., trypanosomiasis) [4,10], or during the wet season, in the presence of a high tick challenge [7]. Furthermore, TBDs also represent a major limitation to the improvement of cattle production given the high morbidity and mortality rates they can cause in more productive, but susceptible, exotic (*Bos taurus*) cattle breeds, when introduced for crossbreeding purposes [13].

Ticks on cattle are perceived as a hazard by the Fulani pastoralists, who traditionally control them by manual removal three times a week during the wet season (i.e., April to October) and twice a week during the dry season (i.e., November to March) [6,10]. Neither dip tanks nor acaricides have ever been used in this part of the country [10].

Knowledge of tick distribution is an essential prerequisite for devising any effective control of these arthropods and the infections they transmit [14]. Existing information on tick infestation of cattle in Nigeria is rather out-dated [5,7,9,15,16], mostly derived from studies carried out in the south of the country [5,15]. The only work published to date on central Nigeria focused on the seasonal dynamics of *Amblyomma variegatum*, without identifying the other specimens retrieved any further than the genus level [7]. West African cattle are currently threatened by the expansion of the harmful and invasive tick species, *Rhipicephalus* (*Boophilus*) *microplus*, seemingly imported from Brazil and found so far only in the Ivory Coast and Benin [17]. Ascertaining the distribution of *Rh. (Bo.) microplus* in this area is important, as this species is the vector of the bovine pathogen *Babesia bovis* [18], and is also resistant to acaricides [19].

The aim of the present work was to document the tick species infesting cattle in central Nigeria, assessing the infestation rate of surveyed animals, at a time of the year (i.e., wet season) when the tick load on the host is known to be most abundant [7].

Methods

Study area

The study was carried out in the second half of September 2010 in 9 villages belonging to three neighbouring local government areas (LGAs), namely Bokkos, Mangu, and Pankshin, in the central part of Plateau State, Nigeria (Figure 1). The study area covered 142 km^2, ranging between latitude 9°14′ and 9°59′ N and longitude 8°79′ and 9°38′ E at an average altitude of 1280 m. All villages are in the sub-humid region of Nigeria, with the dry season generally extending from November to April, and the wet season from April-May to October. The rainfall pattern is mono-modal, with most (~80%) of the rains occurring between June and September. Annual rainfall is ~1400 mm and the daily mean temperature ranges between 18 and 22°C [20].

All cattle reared in the area are of autochthonous (*B. indicus*) genotype, with the vast majority (~80%) belonging to the White Fulani breed, and with a small number of either Bunaji or White Fulani x Bunaji crossbreeds. Cattle are grazed on communal pastures year-round according to the traditional Fulani herding system. Other livestock species raised in the area include sheep, goats, poultry, and dogs. No chemical acaricides are used under the Fulani farming system.

Tick sampling and identification

In each village, all visible adult ticks were collected from at least 15 randomly selected cattle varying in age and sex, all belonging to the indigenous (*B. indicus*) White Fulani breed. Tick collection was performed using blunt steel forceps, by thorough examination of the entire body surface of the animals. Ticks from each animal were stored separately in vials containing 70% ethanol, labelled with information on the host (i.e., sample number, age), village, and date of sampling. Age of the animals was estimated on the basis of the dentition score method developed for zebu cattle under a low plane of

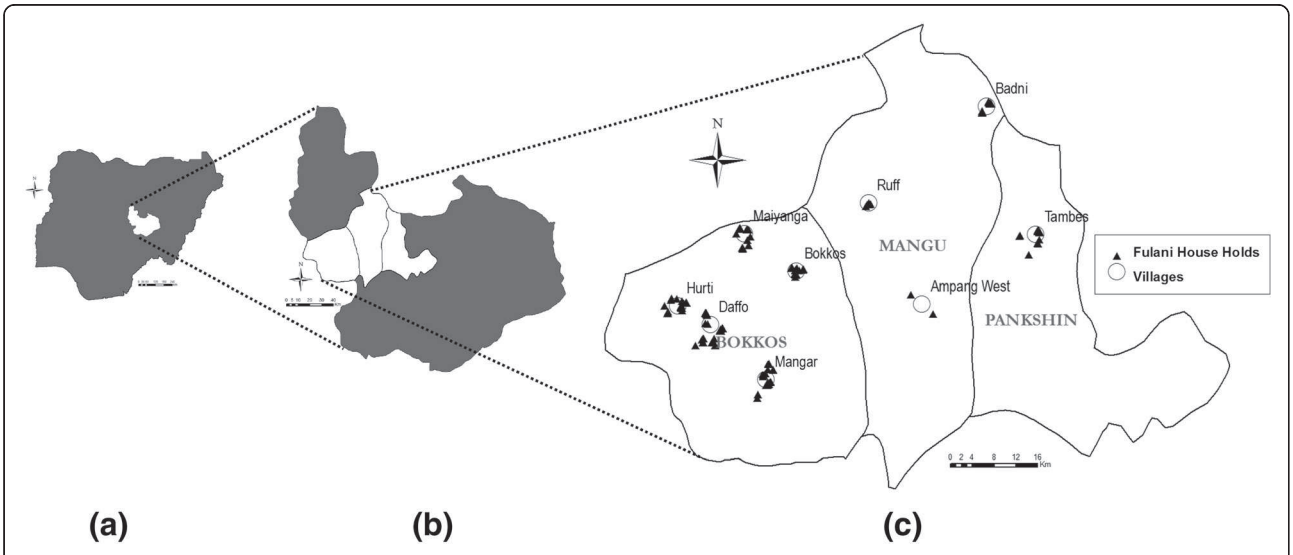

Figure 1 Map of the study area. (**a**): Federal Republic of Nigeria; (**b**): Plateau State; (**c**): local government areas with the 9 study villages.

nutrition [21] and on information provided by their owners. Once quantified, each animal's age was recorded either as 'calf' (0–6 months), 'juvenile' (6–24 months), or 'adult' (older than 24 months). Once in the laboratory, all collected ticks were counted and identified to the genus and species level using a stereomicroscope (up to 100× magnification) and following the morphological keys in Walker *et al.* [22]. For those belonging to the genus *Rhipicephalus*, keys by Walker *et al*. [23] were also used.

Statistical analysis

Statistical analysis was performed using R software (http://www.r-project.org). Prevalence of each tick species was calculated with the exact binomial 95% confidence interval using the reciprocal of the sample size, with the 'survey' package in R. Confidence intervals were calculated by the use of the svymean() function and adjusted with the svyciprop() function. Mean tick counts and their standard error (± SE) were calculated for each village, age group, and tick species. Cumulative counts were statistically compared according to age groups of cattle using the Kruskal-Wallis test. Post hoc analysis was then performed using the Holm P value adjustment method in a pairwise Wilcoxon rank sum test. P values <0.05 were considered statistically significant.

The study was carried out with the full approval of cattle keepers and the federal government body, the Nigerian Institute for Trypanosomiasis Research (NITR). The University of Edinburgh is a charitable body, registered in Scotland, with registration number SC005336.

Results

A total of 228 cattle were checked for tick infestation in 9 villages (average = 25 animals/village). The population sampled consisted of 14 calves, 33 juveniles, and 181 adults. All animals were infested with adult ticks. A total of 5011 adult ixodid ticks (1935 males, 3076 females) were collected (Table 1). Mean tick counts recorded per village were relatively high (i.e., 22 ± 1.4), ranging from 7.6 (±1.5) in Ampang West to 46.5 (±7.91) in Mangar (Table 1). Three tick genera (i.e., *Amblyomma*, *Hyalomma*, *Rhipicephalus* including *Boophilus* sub-genus) and 11 species were identified. *Rhipicephalus* (*Boophilus*) *decoloratus* (Koch, 1844) was the most prevalent species (41.4%), followed by *Rhipicephalus* (*Boophilus*) *annulatus* (Say, 1821) (15.4%); *Rhipicephalus guilhoni* Morel & Vassiliades, 1963 (12.0%); *Rhipicephalus* (*Boophilus*) *geigyi*

Table 1 Cumulative tick counts, mean tick loads ± standard error (SE) according to the villages sampled

Village name	No. of cattle sampled	Total cattle population	No. of ticks collected	Mean tick count/ animal ± SE
Ruff	15	154	322	21.5 ± 3.65
Mangar	15	1373	697	46.5 ± 7.91
Tambes	16	854	301	18.8 ± 4.4
Daffo	21	2933	686	32.7 ± 5.6
Ampang West	22	790	168	7.6 ± 1.5
Hurti	23	1011	594	25.8 ± 4.3
Badni	27	383	851	31.5 ± 3.3
Bokkos	36	2142	608	16.9 ± 2.1
Maiyanga	53	2543	784	14.8 ± 2.4
Total	**228**	**12183**	**5,011**	**22 ± 1.4**

(Aeschliman & Morel, 1965) (7.6%); *Hyalomma truncatum* Koch, 1844 (7.4%); *Amblyomma variegatum* (Fabricius, 1794) (6.3%); *Rhipicephalus simus* Group Koch, 1844 (4.0%); *Rhipicephalus turanicus* Pomerantsev, 1936 (1.2%); *Rhipicephalus sanguineus* (Latreille, 1806) (0.3%); *Hyalomma rufipes* Koch, 1844 (0.2%). Only one male specimen of *Rhipicephalus lunulatus* Neumann, 1907 was retrieved. 4.1% of adult specimens were identified as *Rhipicephalus* (*Boophilus*) spp. but damaged mouthparts prevented identification any further than the sub-genus level (Table 2). *Rh.* (*Bo.*) *microplus* (Canestrini, 1888) was not found in the study area. All three boophilids, *H. truncatum*, and *A. variegatum* were retrieved in all nine villages. Male specimens outnumbered females for most species except for the boophilids and *Rh. sanguineus* (Table 2). A rather high individual variation was seen in terms of tick load, depending on the age and size of the animals. Calves were found to be significantly less infested than adults ($p = 0.004$), whereas no statistically significant difference was found comparing adults with juveniles ($p = 0.2$). Although not statistically significant ($p = 0.2$), the average proportion of ticks infesting juveniles was higher than the mean loads on calves (Table 3).

The broadest diversity of tick species was encountered in adults, followed by juveniles, and calves. Boophilids, *H. truncatum*, and *A. variegatum* were more abundant in adults compared to juveniles and, more markedly, than calves (Table 4).

Table 2 Cumulative counts, prevalence, number of males and females, and male: female ratio of ticks identified

Tick species	Total	Mean prevalence% (95% confidence interval)	Males	Females	Male: female ratio
Rhipicephalus (*Boophilus*) *decoloratus*	1890	41.4 (36.5–46.3)	473	1417	1 : 3
Rhipicephalus (*Boophilus*) *annulatus*	819	15.4 (11.9–19.0)	189	630	1 : 3.3
Rhipicephalus guilhoni	434	12.0 (7.2–16.8)	302	132	2.3 : 1
Rhipicephalus (*Boophilus*) *geigyi*	306	7.6 (6.1–9.1)	45	261	1 : 5.8
Hyalomma truncatum	681	7.4 (5.8–9.0)	469	212	2.2 : 1
Amblyomma variegatum	361	6.3 (4.5–8.1)	245	116	2.1 : 1
Rhipicephalus (*Boophilus*) spp.	205	4.1 (2.9–5.4)	12	193	1 : 16
Rhipicephalus simus Group	239	4.0 (2.5–5.5)	155	84	1.8 : 1
Rhipicephalus turanicus	39	1.2 (0.5–2.0)	22	17	1.3 : 1
Rhipicephalus sanguineus	10	0.3 (0.03–1.0)	4	6	1 : 1.5
Hyalomma rufipes	26	0.2 (0.1–0.4)	18	8	2.2 : 1
Rhipicephalus lunulatus	1	<0.1 (0.0–0.0)	1	–	1 : 0

Discussion

The distribution of ticks within a specific habitat depends on several environmental and climatic factors such as annual rainfall, atmospheric temperature and relative humidity (RH), vegetation cover, altitude and host availability [24]. This study was carried out in the late wet season, when RH as well as the vegetation coverage, and therefore the abundance of adult ticks on cattle, are expected to be at their peak in central Nigeria [7,9,10]. This study aimed to assess the species diversity of ticks infesting cattle and their burdens; we only focused on the adult stages of these arthropods. Because of their small size, a large number of immature ticks can indeed be easily overlooked during field collection, resulting in a biased estimate of counts. Therefore, counts of adults can be taken as representative of the total infestation of all instars over the year, especially for three-host tick species, whose immature instars feed for short periods (e.g., four days) on cattle as well as on other hosts (e.g., small ruminants, wildlife, birds) [25]. In addition, larvae and nymphs of most genera lack the neatly distinctive morphological features needed for identification to the species level.

An average of 25 randomly selected cattle at each of the 9 villages were examined (Table 1). The greater number of adults rather than younger animals sampled reflects the age composition of Fulani herds, with at least 60% of cattle being adult [10]. The study ascertained the presence of a rather broad variety of tick species infesting cattle in central Nigeria, belonging to three genera (i.e., *Amblyomma*, *Hyalomma*, *Rhipicephalus* spp.) included in the Family Ixodidae. Five out the 11 species identified (i.e., *Rh.* (*Bo.*) *decoloratus*, *Rh.* (*Bo.*) *annulatus*, *Rh.* (*Bo.*) *geigyi*, *H.*

Table 3 Cumulative tick counts and mean tick loads ± SE of cattle according to age groups

Age group	Cumulative counts	Mean tick load ± SE
Calves (<6 months)	142	10.1 ± 2.7[a]
Juveniles (6–24 months)	595	18.0 ± 2.9
Adults (>24 months)	4274	23.6 ± 1.6[a]

[a] Statistical significance between the two age groups.

Table 4 Cumulative counts and mean loads ± SE of tick species according to age groups of cattle

Tick species	Cumulative counts			Mean tick load ± SE		
	Calves	Juveniles	Adults	Calves	Juveniles	Adults
Rhipicephalus (*Boophilus*) *decoloratus*	56	197	1637	4.0 ± 1.2	6.0 ± 1.7	9.0 ± 0.9
Rhipicephalus (*Boophilus*) *annulatus*	16	104	699	1.1 ± 0.5	3.1 ± 0.7	3.9 ± 0.4
Rhipicephalus guilhoni	27	54	353	1.9 ± 1.3	1.6 ± 0.7	1.9 ± 0.6
Rhipicephalus (*Boophilus*) *geigyi*	7	27	272	0.5 ± 0.2	0.8 ± 0.2	1.5 ± 0.2
Hyalomma truncatum	15	87	579	1.1 ± 0.4	2.6 ± 0.8	3.2 ± 0.4
Amblyomma variegatum	12	42	307	0.9 ± 0.4	1.3 ± 0.2	1.7 ± 0.2
Rhipicephalus (*Boophilus*) spp.	1	32	172	0.1 ± 0.1	1.0 ± 0.4	0.9 ± 0.1
Rhipicephalus simus Group	7	33	199	0.5 ± 0.4	1.0 ± 0.5	1.1 ± 0.2
Rhipicephalus turanicus	1	10	28	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1
Rhipicephalus sanguineus	0	7	3	0	0.2 ± 0.2	0.02 ± 0.01
Hyalomma rufipes	0	2	24	0	0.1 ± 0.04	0.1 ± 0.04
Rhipicephalus lunulatus	0	0	1	0	0	0.01 ± 0.01

truncatum, and *A. variegatum*) were retrieved in all study villages.

Rh. (*Bo.*) *decoloratus* was the most abundant species in the area, in accordance with previous work [5]. The Nigerian Jos Plateau seemingly provides an ideal environment for *Rh.* (*Bo.*) *decoloratus*, preferring highlands and sub-highlands receiving more than 800 mm of rainfall annually [26]. The second most prevalent species in this study was *Rh.* (*Bo.*) *annulatus*, previously found to be the most common tick attacking cattle in eastern Nigeria [15]. In Africa, the distribution of this tick is restricted to the northern and western part of the continent [22]. South of the Sahara, *Rh.* (*Bo.*) *annulatus* is associated with lowland rainforest and secondary grassland, with a clear increase in the vegetation cover after July-August [27]. Both *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *annulatus*, transmit *Babesia bigemina* [18], *Anaplasma marginale* and *Anaplasma centrale* [28], known to be endemic in Nigeria [8]. Being boophilids, one-host ticks that entirely develop on cattle after the egg hatch, their population is expected to be relatively constant throughout the year in this setting [9], presenting a constant threat of bovine anaplasmosis and babesiosis.

This study provides the first record of *Rh. guilhoni* in central Nigeria. Small numbers of adults of this tick were previously collected from the cattle during the rainy season in the far north of Nigeria [16]. Here, *Rh. guilhoni* was retrieved in 8 out of 9 villages and was the third most prevalent tick species (Table 2). As members of the *Rh. sanguineus* Group, this species is characterised by a more dense interstitial punctuation in the conscutum and a female genital aperture of a more truncated V-shape than the progenitor of its taxonomical group [23]. It is usually found infesting cattle, sheep, and camels, in steppe and savanna climatic regions [23]; its considerable presence on the Jos Plateau highlights the importance of assessing its role in pathogen transmission, as yet unknown.

This study also identified *Rh.* (*Bo.*) *geigyi* in central Nigeria. This species, only present in West Africa, is normally found in the savanna and forest zones of southern Nigeria, where it is the most abundant boophilid in the early dry season [5]. As this tick requires higher mean temperatures than *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *annulatus* [27], it would be expected that the cooler conditions of the Plateau and, more in general, of the northern Guinea savanna woodland, would limit the expansion of its population into central-northern Nigeria. Although little studied in terms of pathogen transmission, *Rh.* (*Bo.*) *geigyi* could be of veterinary relevance in Nigeria, where it was proven to harbour piriform kinetes associated for shape and size with *B. bovis*, in both eggs and larvae that eventually infected splenectomised calves [29].

A number (n = 205) of boophilids were identified only as *Rhipicephalus* (*Bo.*) spp. due to partial rupture of their mouthparts, likely to have occurred at the time of collection, considering the small size and the short rostrum of these ticks. In particular, these were mostly engorged female specimens (see Table 2), whose feeding state did not allow the objective assessment of morphological features (e.g., shape of genital aperture), other than the mouthparts. The rostrum of boophilids bears species-specific features, such as the teeth rows in the hypostome and palp articles [22]. Nevertheless, as all these specimens had either one or both palps bearing a protuberance with or without an intact pectinate seta on article I, it was still possible to rule out the presence of *Rh.* (*Bo.*) *microplus* amongst them. The damage in their hypostome, though, did not allow the discrimination

between *Rh.* (*Bo.*) *decoloratus* and *Rh.* (*Bo.*) *geigyi*, thus limiting the definitive identification to the sub-genus level.

The high number (n = 681) of *H. truncatum* recorded, reflects the seasonality of this tick in Nigeria, where it is known to peak in the late wet season [7,9]. The veterinary importance of this species is related to its ability to cause a toxic syndrome (sweating sickness), especially in young cattle [30].

In a study carried out in the neighbouring state of Kaduna, central Nigeria, *A. variegatum* was the most prevalent species (>80% of all collected ticks) parasitizing cattle in September, followed by *Rhipicephalus* (*Bo.*) spp., and *Hyalomma* spp. [7]. The lower prevalence of *A. variegatum* (6.3%) recorded in the present study could be mainly attributed to the practice of hand-picking of ticks by the Fulanis, carried out up to three times a week during the wet season [6]. This control method mainly targets the most conspicuous *Amblyomma* adults, regarded as 'koti' (i.e., 'dangerous ticks' in Fulfulde language), by the local herdsmen, as opposed to the smaller *Rhipicephalus* and boophilid ticks that are consciously left attached, as they are believed to be 'miri' (i.e., 'less harmful') [7]. This operation is carried out when the animals are standing, when a number of body areas (e.g., groin, hooves, etc.) of the cattle cannot be easily reached; *H. truncatum* adults, that preferentially localize in the inter-digital clefts and the tail switch [31,32], are frequently overlooked (see Figure 2). In addition, although it keeps the animal to some extent free from 'tick worry', hand removal of ticks may not prevent transmission of tick-borne infections when not performed on a daily basis, as the transmission of pathogens may occur two days after the attachment of these arthropods to their hosts [33]. Due to their long mouthparts, *A. variegatum*, as well as *Hyalomma* ticks, can inflict serious cutaneous damage to cattle. Importantly, due to their preferential attachment to the udder and teats of cattle [31,34-37], infestation by both these tick genera may seriously hinder the suckling of calves. *A. variegatum* is of veterinary importance as it transmits *Ehrlichia* (*Cowdria*) *ruminantium* [38], causative agent of heartwater and *Dermatophilus congolensis*, causing dermatophilosis [39], both known to be endemic in Nigeria. *A. variegatum* is also a vector of the mildly pathogenic, *Theileria mutans* [40,41] and *Theileria velifera* [42] both highly prevalent in Nigeria.

The paucity (n = 26) of adult *H. rufipes* collected in this study could indicate a small population of this species, known to be widely distributed in the most arid parts of tropical Africa [43]. Adults of *H. rufipes* are usually more numerous in the early part (i.e., June-July in Nigeria) than towards the end of the rainy season [44]. It is also possible that the altitude of the Jos Plateau might have acted as a further limiting factor to the establishment of an *H. rufipes* population. Considering that both larvae and nymphs of this two-host tick parasitize ground-feeding birds [45], it is likely that the adult specimens came from the moult of engorged nymphs brought to the study area by birds living in close contact with the herds (e.g., cattle egrets, oxpeckers, guinea fowls, etc.). Interestingly, no *H. rufipes* ticks were collected from calves (Table 4), suggesting that open pastures, grazed mainly by adult cattle, represent the most likely interface between cattle and these birds. Although scanty, the presence of this tick species is still of veterinary importance as it is known to transmit *A.*

Figure 2 Young Fulani herders from the Plateau removing ticks manually from their cattle.

marginale [46], *Theileria annulata* [47], and *Babesia occultans* [48] to cattle. A relatively high number (n = 239) of adult *Rh. simus* Group ticks were collected from cattle of all age groups. Of the three taxa currently ascribed to the '*simus* Group', only *Rhipicephalus muhsamae* Morel & Vassiliades (1965) was expected to be present in West Africa. However, in addition to the Group-specific punctuation pattern visible on the males' conscutum, a number of morphological features (e.g., female genital aperture and shape of adanal plates) of the specimens collected in this study appeared closely related to the East African taxon *Rhipicephalus praetextatus* Gerstäcker, 1873. It was assumed these were the same specimens retrieved in the 1950s from several localities in central and northern Nigeria, identified as *Rhipicephalus simus simus* [49]. Usually found in regions with a savanna climate, the distribution of *Rh. simus* (or *Rh. simus simus*) is believed to be restricted to southern Africa [26], where the adults preferentially parasitize cattle, never reaching high loads [50]. As *Rh. simus* is a vector of *A. centrale* [51] and *A. marginale* [52] usually found in southern Africa, finding these specimens in Nigeria may be of epidemiological importance.

Other *Rhipicephalus* species collected include *Rh. sanguineus, Rh. turanicus*, and *Rh. lunulatus*. *Rh. sanguineus* in cattle has previously been recorded elsewhere [23,53] including Nigeria [15,54,55] and can be related to the presence of dogs, roaming freely within the boundaries of the villages where sampling took place, and in the vicinity of the cattle herds. This cosmopolitan three-host tick species is always associated with dogs, its preferential host, and the human-made dwellings where they live [56]. *Rh. turanicus* is usually more adapted to sheep and goats rather than cattle [57], and this might explain the small number (n = 39) of specimens collected in our study. Both *Rh. sanguineus* and *Rh. turanicus* are not known to transmit any pathogens to cattle [58]. Interestingly, only one male specimen of *Rh. lunulatus* was identified; this species has very distinctive morphological features (e.g., adanal plates' shape in males; very broad U-shaped genital aperture in females) [59] compared to the other *Rhipicephalus* spp. ticks found in this survey. Adults of this three-host tick were previously reported in cattle in northern Nigeria, where they were found only during the first half of the wet season [16,49]. *Rh. lunulatus* is not regarded as a hazardous tick for cattle, although it was associated with a toxicosis causing paralysis in calves in Zimbabwe [60].

Rh. (*Bo.*) *microplus* was not found in the study area. We therefore assume that the eastbound expansion in West Africa of this invasive tick species, found first in 2007 in the Ivory Coast, and then in Benin [17], has not yet reached central Nigeria. This is of great epidemiological interest, as this tick species primarily parasitizing cattle, is known for being the competent vector of the highly pathogenic *B. bovis* [18]. Furthermore, the absence of *Rh.* (*Bo.*) *microplus* is also of interest in terms of tick control management, as this species is known to be highly resistant to several pyrethroid and organophospate compounds [19].

Males constituted the majority of specimens collected for most species (i.e., *Amblyomma, Hyalomma,* and *Rhipicephalus* spp.), with the exception of *Rh. sanguineus* and *Boophilus* spp. (Table 2). The male:female ratios recorded for most ticks coincide with data from previous work, with special reference to *Rh.* (*Bo.*) [25,61-64], *A. variegatum* [37,61,62,64], *H. rufipes* [62], but not for *Rh. sanguineus* [65]. With reference to *A. variegatum* the higher proportion of males rather than females collected is attributable to the biology of this tick species, known for localizing in preferential body areas (e.g., armpit, groin, udder, scrotum), forming typical clusters including a few females clasped by several males [31,32]. This is due to the release of aggregation-attachment pheromones (AAP) produced only by *A. variegatum* males, attracting unfed males and females [66] resulting in a concentration of more males than females on the attachment sites. The greater number of males than females collected for *Rhipicephalus* spp. is probably due to the fact that fully engorged female ticks are more easily groomed by the animals [67] and also drop to the ground earlier to lay eggs, while males tend to remain on the host for longer periods, feeding and mating several times before dropping-off [65]. This biological feature has been well documented for *Rh. sanguineus* [67], although in this study more females than males were collected, with a very low cumulative count (n = 10). The higher number of female rather than male boophilids collected is consistent with other studies [62] and likely reflects the relative difficulty in collecting the smaller males from hosts.

Here, the overall mean tick load recorded (i.e., 22 ± 1.4) was considered to be relatively high in the light of the hand-picking practice described above, which most likely reduced the actual number of adult ticks on the cattle sampled. It is also possible that the transhumance of weaned cattle according to the traditional Fulani herding might play a role in containing tick burdens as grazing areas are naturally spelled. Nevertheless, all the most hazardous tick species were recorded, although with different abundances (see Table 4), in all age groups in all study villages, with potentially large implications in terms of pathogen transmission.

In particular, this study revealed a pronounced effect of host age and size on the number of infesting adult ticks, especially when comparing calves (< 6 months) with adult cattle (>24 months of age) (Table 3). Although with no statistical significance, the mean tick loads of calves were also found at a lower proportion than those of juvenile cattle (6–24 months old), which bore lower burdens

than the adults (Table 3). This finding is of interest considering that a significant amount of calving in the Fulani herds takes place in the early wet season (April-May) [10] and therefore, the calves sampled in this study have likely lived through the entire rainy season, in the presence of a high tick challenge. The significantly lower tick loads observed in calves as opposed to adults corroborates similar work carried out on indigenous cattle in SSA [68,69] including Nigeria [15,70]. The lower tick burdens recorded in calves could be indeed due to a combination of factors, including some form of innate immunity of indigenous cattle that decreases with age [71], the persistent grooming of calves by their respective dams [72], and the smaller body surface of younger animals compared to adults [73]. It could be argued, that animals with larger surface areas would possibly allow more contact opportunities for the ticks to attach themselves. This is also predicted by body size principle, according to which, the smaller the animal the fewer parasites (i.e., engorging ticks) it can afford to accumulate per unit of body surface because of the greater body surface to mass ratio [73]. Moreover, the lower tick burden recorded in young animals could also be due to the Fulanis' practice of maintaining calves tethered together close to the homesteads, separated from the adult cattle. They therefore spend limited time grazing in the open grasslands with their dams, being possibly less exposed to the higher parasite burdens found on the pastures, driven by the higher host density.

Conclusions

This study provides new information on tick populations in Nigeria and, more globally, in West Africa. The finding of *Rh.* (*Bo.*) *decoloratus*, *Rh.* (*Bo.*) *annulatus*, *A. variegatum*, and *H. truncatum* in all study villages is of great veterinary importance as these species are involved in the transmission of anaplasmosis, babesiosis by *B. bigemina* (*Boophilus* spp.); cowdriosis and dermatophilosis (*A. variegatum*) and sweating sickness (*H. truncatum*) [3]. Further studies are necessary to assess the occurrence of related TBDs in the Plateau State and would also help address the possible introduction of exotic breeds into the area.

Rh. (*Bo.*) *microplus* was not found in the present study, suggesting that this invasive and hazardous tick is not yet established in central Nigeria. Constant monitoring would, however, be advisable, as the Nigerian Jos Plateau provides favourable climatic and environmental conditions for the establishment of this tick species [27].

All animals sampled in this study were found infested with relatively high tick burdens. In order to be effectively implemented in the Jos Plateau, any strategic tick control should take into account the traditional farming system of the Fulani pastoralists. This could be achieved by combining the long-employed practice of manual removal of ticks with conventional control methods (i.e., acaricides) during the wet season when tick loads peak. In particular, in the light of inefficiency of the hand-picking method and that most of the calving takes place at the end of the dry season [10], the implementation of minimal or threshold tick control for adult female cattle, based on the application of spray or 'pour-on' acaricides to the udder region, might help prevent the topical attachment of *A. variegatum* and *Hyalomma* ticks, thereby improving milk yields [25]. Importantly, such a strategy would also preserve the endemic stability of the indigenous cattle herds in the area with regards to bovine TBDs [74]. Furthermore, while the indigenous White Fulani cattle are better able to bear these tick burdens, it is likely that exotic (*B. taurus*) or cross (*B. indicus* x *B. taurus*) breeds, if introduced in this area, unless subjected to intensive acaricide treatment, will become heavily infested with ticks and exposed to TBDs.

Consent

The photograph was taken with the consent of the individuals portrayed and their families, as well as the community chief in the village of Maiyanga, Bokkos local government area.

Competing interests

The authors declare they have no competing interests and the sponsors had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

VL, KP, AM, AI, and SCW conceived of the study and participated in its design. AM coordinated the field activities. VL, GB, and CD carried out the tick collection. VL took care of tick identification. VL and BMCB carried out the statistical analysis. VL, KP, BMCB, and SW wrote the paper. All authors read and approved the final manuscript.

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are needed to determine the role of nasal carriage in *B. holmesii* bacteremia. That no *B. holmesii* infections occurred after rituximab was stopped suggests that rituximab played a role in the recurrent infections. In cases of recurrent infection or bacteremia, nasal carriage should be assessed, and the interruption of rituximab should be considered by physicians.

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Rickettsia africae in *Amblyomma variegatum* Ticks, Uganda and Nigeria

To the Editor: *Rickettsia africae* is the most widespread spotted fever group (SFG) rickettsia in sub-Saharan Africa, where it causes African tick-bite fever (*1*), an acute, influenza-like syndrome. The number of cases in tourists returning from safari in sub-Saharan Africa is increasing (*1*). In western, central, and eastern sub-Saharan Africa, *R. africae* is carried by *Amblyomma variegatum* (Fabricius, 1794) ticks (*2*); usually associated with cattle, this 3-host tick also can feed on a variety of hosts, including humans (*2*). *R. africae* has not been reported in Uganda and rarely reported in Nigeria (*3*,*4*). Our objective was to determine the potential risk for human infection by screening for rickettsial DNA in *A. variegatum* ticks from cattle in Uganda and Nigeria.

In February 2010, ticks were collected from zebu cattle (*Bos indicus*) from 8 villages in the districts of Kaberamaido (Adektar [1°81′N–33°22′ E], Awimon [1°66′N–33°04′ E], Kalobo [1°88′ N–33°25′ E], Odidip [1°90′ N–33°30′ E], Odikara [1°91′ N–33°30′ E], and Olilimo [1°75′ N–33°38′ E], and Dokolo (Alela [2°09′ N–33°16′ E], and Angeta [1°87′ N–33°10′ E]) in Uganda and, in June 2010, in 3 villages (Mangar [9°14′ N–8°93′ E], Ruff [9°43′ N–9°10′ E], and Tambes [9°38′ N–9°38′ E]) in the Plateau State in Nigeria (Figure). This convenience sample was obtained as part of other ongoing research projects in both countries. Ticks were preserved in 70% ethanol and identified morphologically to the species level by using taxonomic keys (*5*). Because the anatomic features do not enable an objective assessment of the feeding status of adult male ticks, engorgement level was determined only in female tick specimens and nymphs.

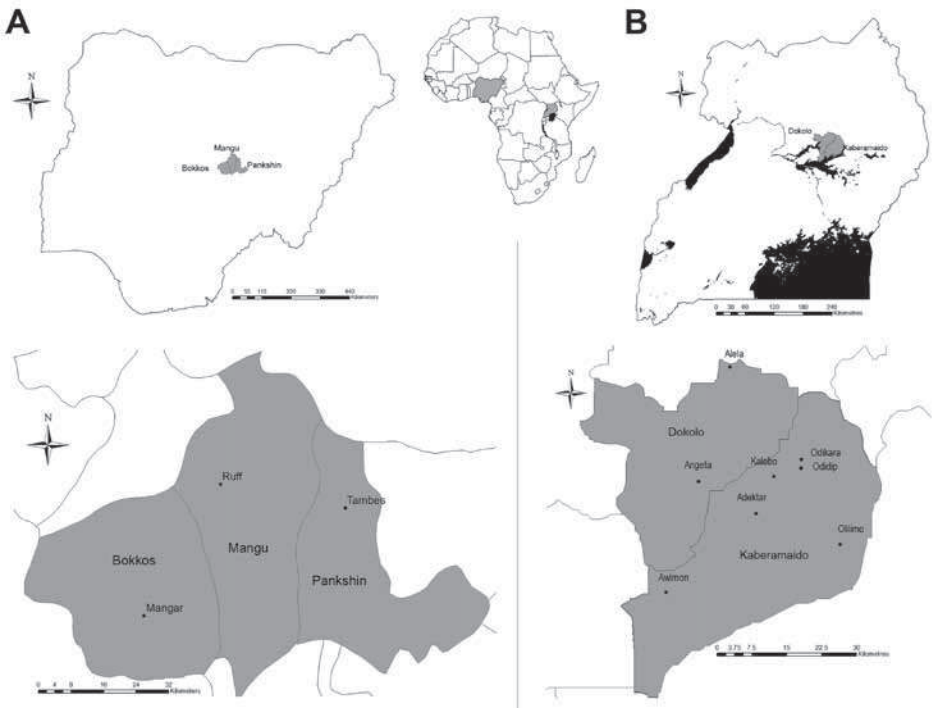

Figure. Location of areas studied for *Rickettsia africae* in *Amblyomma variegatum* ticks in Nigeria (A) and Uganda (B), 2010.

After tick identification, DNA was extracted from ticks by using QIAmp DNeasy kits (QIAGEN, Hilden, Germany). Two PCR targets were assessed within each sample; the primer pair Rp.CS.877p and RpCS.1258n was selective for a 396-bp fragment of a highly conserved gene encoding the citrate synthase (*glt*A) shared by all *Rickettsia* spp. (*6*); the Rr190–70p and Rr190–701n primer pair amplified a 629–632-bp fragment of the gene encoding the 190-kD antigenic outer membrane protein A (*omp*A), common to all SFG rickettsiae (*6*,*7*). DNA extracted from 2 *A. variegatum* tick cell lines (AVL/CTVM13 and AVL/CTVM17), previously amplified and sequenced by using primers for *Rickettsia* 16S rRNA, *omp*B, and *sca4* genes revealing >98% similarity with *R. africae* (*8*), was used as a positive control. Negative controls consisted of DNA from 2 male and female laboratory-reared *Rhipicephalus appendiculatus* ticks and distilled water. DNA of positive samples was recovered, and confirmation of amplicon authenticity was obtained through sequence analysis by using nucleotide BLAST (www.ncbi.nlm.nih.gov/BLAST).

A total of 39 ticks were collected in Uganda (32 adult males, 5 females, and 2 nymphs), and 141 were collected in Nigeria (80 males, 59 females, and 2 nymphs); all were identified as *A. variegatum* (online Technical Appendix Table, wwwnc.cdc.gov/EID/articlepdfs/19/10/13-0389-Techapp1.pdf). SFG rickettsiae DNA was amplified in 26 (67%) of 39 ticks from Uganda and 88 (62%) of 141 ticks from Nigeria by using the *omp*A gene primers; amplicons of the *glt*A genes were obtained in 16 (41%) of 39 ticks and 84 (60%) of 141 ticks, respectively (online Technical Appendix Table). Overall, 81 (45%) of 180 ticks were positive by *glt*A and *omp*A PCRs (online Technical Appendix Table). DNA sequences of the 22 *glt*A and *omp*A products from Uganda and the 22 from Nigeria showed 100% similarity with published sequences of *R. africae* (GenBank accession nos. U59733 and RAU43790, respectively). For both countries, ticks positive for *Rickettsia* spp. and SFG rickettsiae DNA were male and female specimens (online Technical Appendix Table). Among females, both unengorged and engorged specimens contained DNA from rickettsiae and SFG rickettsiae (online Technical Appendix Table).

These findings represent a novelty for Uganda. With reference to Nigeria, our results contrast with the prevalence of 8% recorded in a similarly sized sample (n = 153) of *A. variegatum* ticks collected from cattle in the same part of the country (*3*); this discrepancy might be the result of previous targeting of the rickettsial 16S rDNA gene. In the study reported here, the SFG-specific *omp*A PCR proved to be more sensitive than *glt*A for detecting rickettsiae DNA, as has also been reported in previous work (*9*). Although finding *R. africae* DNA in engorged female and nymphal tick specimens might be attributable to prolonged rickettsemia in cattle (*10*), the presence of *R. africae* in distinctly unengorged female ticks indicates the potential for *A. variegatum* ticks to act as a reservoir of this SFG rickettsia (*2*).

This study extends the known geographic range of *R. africae* in *A. variegatum* ticks in sub-Saharan Africa. The number of potentially infective ticks recorded in Uganda and Nigeria suggests that persons in rural areas of northern Uganda and central Nigeria might be at risk for African tick-bite fever. Awareness of this rickettsiosis should be raised, particularly among persons who handle cattle (e.g., herders and paraveterinary and veterinary personnel). Physicians in these areas as well as those who care for returning travelers, should consider African tick-bite fever in their differential diagnosis for patients with malaria and influenza-like illnesses.

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Ongoing Measles Outbreak in Orthodox Jewish Community, London, UK

To the Editor: Measles outbreaks have been reported in Orthodox and ultra-Orthodox Jewish communities across Europe and Israel (*1*–*5*). We describe an ongoing outbreak within the largest European Orthodox Jewish community (including a Charedi population of 17,587), based in London, focused in Hackney (*6*). Vaccination coverage within this community is lower than in the general population of London, causing low herd immunity and outbreaks of vaccine-preventable diseases. Vaccination coverage data within the communities cannot be extrapolated, because membership is not classified as an ethnicity and not collected within health electronic recording systems. However, general practice surgeries in Hackney known to have high proportions of Orthodox Jewish patients have considerably lower vaccination coverage (55%–75% of patients 24 months of age had received measles, mumps, rubella [MMR] vaccine in the 3rd quarter of 2012) compared with the London average (87.3%) (*7*). Health beliefs, family size (the average Charedi household size is 6.3 persons), and underutilization of immunization services contribute to low coverage (*8*,*9*).

The outbreak clinical case definition was taken from Public Health England's guidance (*10*). It also included membership in the Orthodox

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